# Plant Food Allergens

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*Edited by*

E.N. CLARE MILLS
*Institute of Food Research*
*Norwich, UK*

PETER R. SHEWRY
*Rothamsted Research*
*Harpenden, UK*

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# Contributors

**Alicia Armentia**
Sección de Alergia, Hospital Universitario Río Ortega, Valladolid, Spain

**Gary A. Bannon**
Product Safety Centre, Monsanto, St Louis, Mo, USA

**Domingo Barber**
Departamento de I+D, ALK-Abelló, Madrid, Spain

**Frederic Beaudoin**
Rothamsted Research, Harpenden, Herts, AL5 2JQ, UK

**Carlos Blanco**
Sección de Alergia, Hospital de Gran Canaria Dr. Negrín, Las Palmas de Gran Canaria, Spain

**René Crevel**
Safety and Environmental Assurance Centre, Unilever, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

**Paul J. Davis**
CEO, Insense Ltd, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

**Araceli Díaz-Perales**
Unidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Madrid, Spain

**Jean-Paul Douliez**
Institut National de la Recherche Agronomique, Unité de Biochimie et Technologie des Protéines BP71627, 44316 Nantes cedex 03, France

**Khalil Elmorjani**
Institut National de la Recherche Agronomique, Unité de Biochimie et Technologie des Protéines BP71627, 44316 Nantes cedex 03 and 2, France

**Montserrat Fernández-Rivas**
Unidad de Alergia, Fundación Hospital Alcorcón, Madrid, Spain

**Gloria García-Casado**
Unidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Universidad Politécnica, Madrid, Spain

**Marie-Françoise Gautier**
Unité de Biochimie et Biologie Moléculaire des Céréales, 2 Place Viala, 34060 Montpellier cedex 01, France

**Luis Gomez**
Unidad de Química y Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros de Montes, Universidad Politécnica, Madrid, Spain

**Karin Hoffmann-Sommergruber**
Department of General and Experimental Pathology, University of Vienna, AKH, EBM-3Q, A-1090 Austria

**John A. Jenkins**
Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, UK

**Charlotte Madsen**
Department of Toxicology, Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration, 19, Mørkhøj Bygade, DK-2860 Søborg Denmark

**Didier Marion**
Institut National de la Recherche Agronomique, Unité de Biochimie et Technologie des Protéines BP71627, 44316 Nantes cedex 03, France

**Ariette Matser**
Agrotechnological Research Institute (ATO), Bornsesteeg 59, 6708 PD Wageningen, The Netherlands

**Susan Miles**
Research Associate, School of Medicine, Health Policy and Practice, University of East Anglia, Norwich, NR4 7TJ, UK

**E.N. Clare Mills**
Institute of Food Research, Norwich Research Park, Colney Lane, Norwich, NR4 7UA, UK

**Rafael I. Monsalve**
Departamento de Bioquímica, Facultad de Químicas, Universidad Complutense, Madrid 28040, Spain. Present address: ALK-Abellóm Calle Miguel Fleta 19, Madrid 28037, Spain

**Christian Radauer**
Department of General and Experimental Pathology, University of Vienna, AKH, EBM-3Q, A-I090 Austria

**Manuel Rico**
Instituto de Química Física "Rocasolano", Consejo Superior de Investigaciones Científicas (CSIC), Madrid 28006, Spain

**Rosalía Rodríguez**
Departamento de Bioquímica, Facultad de Químicas, Universidad Complutense, Madrid 28040, Spain

**Gabriel Salcedo**
Unidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Universidad Politécnica, Madrid, Spain

**Rosa Sánchez-Monge**
Unidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Universidad Politécnica, Madrid, Spain

**Peter R. Shewry**
Rothamsted Research, Harpenden, Herts, AL5 2JQ, UK

**Cristina Soler-Rivas**
Agrotechnological Research Institute (ATO), Bornsesteeg 59, 6708 PD Wageningen, The Netherlands

**Aart van Amerongen**
Agrotechnological Research Institute (ATO), Bornsesteeg 59, 6708 PD Wageningen, The Netherlands

**Mayte Villalba**
Departamento de Bioquímica, Facultad de Químicas, Universidad Complutense, Madrid 28040, Spain

**Harry Wichers**
Agrotechnological Research Institute (ATO), Bornsesteeg 59, 6708 PD Wageningen, The Netherlands

**Jan Wichers**
Agrotechnological Research Institute (ATO), Bornsesteeg 59, 6708 PD Wageningen, The Netherlands

# Preface

## Historical and Cultural Background to Plant Food Allergies

PAUL J. DAVIS

This book is concerned with a paradox of immense, potentially life-threatening significance to about 1 in 100 adults and nearly 1 in 10 children, within the European Union. The paradox is that certain nutritious proteins from wholesome foods can act as if they were harmful, sometimes deadly, poisons to otherwise normal people unfortunate enough to possess a food allergy. And, although immunologists have been occupied with the whole problem of allergy throughout the twentieth century, we enter the twenty-first century with a vast number of questions still unanswered. Whilst the lives of food-allergic patients have certainly been improved through the understanding gained so far, an allergic individual is still haunted by the ever-present threat of inadvertent exposure to the allergen – an event that could have devastating consequences. The impact of this constant fear on the lives of caring parents with a food-allergic child, for example, is immense, and can only be appreciated by those who have experienced at first hand the torment involved. There can be no doubt that food allergy is a major, unsolved problem of great economic, social and personal significance. And the problem is getting even worse, because of the current trend in the developed world for an increasing diversity of foods to be routinely available, with an ever-increasing variety of complex recipes and formulations.

So, there is still much to do, and the EU-funded European network, called PROTALL, brought together over 30 scientists with a particular blend of expertise relevant to studying the complex problems of food allergy. In order to shed new light on this persistent problem, the network deliberately combined the diverse insights of clinicians, food scientists and plant biologists, with a focus on the relationship between the allergenic potential of plant food proteins, their molecular structures, their biological activities, their processing history and their interactions with other food components, such as fats. Such diverse groups of experts are rich sources of creative insights and fresh thinking – a far cry from the old, restricted membership of the New York *Allergy Roundtable Discussion Group* founded between the World wars. Typical of the attitudes of the time, this august and ground-breaking body was restricted entirely to clinicians until 1949, when the first basic scientist, Merrill W. Chase, PhD, was admitted!

The PROTALL network concluded at the end of 2000, and this book is largely based on the outcome of its investigations. Whilst the literature is rich with information on the topic of food allergy, this is the first book to present a coherent account of plant proteins as allergens to humans. It begins to make sense of why some types of proteins are more allergenic than others, and provides a unique source of information on particular groups of proteins in relation to their structure, function and phylogenetic relationships. These insights can bring us closer to the elusive but much to be prized ability to predict the allergenicity of food proteins. The mysteries are gradually being unravelled by identifying key physicochemical properties common to known allergens and by working out which particular chemical structures (epitopes) are

recognised by immunoglobulin E (IgE) antibodies. These types of study have already shown that if a protein is stable through processing (e.g. acid and heat-treatments) and resistant to digestion, it is more likely to be an allergen than one that is easily degraded.

Usually, food allergy research has (for very good reasons) involved the use of purified proteins, following the typical reductionist approach that helps to make sense of complex problems. But now it is time to move on, for this grossly simplified situation does not resemble that which results from the ingestion of *food as eaten*, when complex mixtures of proteins, usually in a highly processed form, interact together and with other food components during cooking, eating and digestion. For example, lipid-binding proteins will normally be associated with lipids when they occur within food, rather than being in a free state or dissolved in water. For this reason, the PROTALL studies have included investigations of the interactions and fate of key proteins within realistic mixtures and processes, thus getting to grips with what actually happens in food.

It is important to appreciate that the allergic reactions studied in this project relate to the most common and easily diagnosed food allergies, known as Type I-hypersensitivity reactions. It includes neither the less clear-cut condition defined as *food intolerance*, nor those adverse reactions caused by other immunological and toxicological mechanisms. Type I-hypersensitivity reactions are mediated by IgE antibodies, and have a rapid onset and, usually, a brief duration. Judged by the vanishingly small amounts of IgE in the serum, IgE would have seemed an insignificant player in the complex drama of the immune system. But the low serum levels of IgE are very deceptive, for most of the IgE in the body is to be found on the surface membranes of the vast population of mast cells in the tissues and basophils in the blood, held in place by specific receptors. More dramatically, the true significance can be judged by the overall effects on the whole body.

So what are these effects, usually summarised rather blandly as the *allergic reaction*? It was Carl Prausnitz and Heinz Kustner, two clinicians at the Institute of Hygiene in Breslau, who first began to make the link between an undetectable serum component and the symptoms of food allergy (termed by them *supersensitivity*), when they published in 1921 a remarkably insightful paper that brought about a step change in understanding [1]. They were intrigued by the observation that Kustner was *supersensitive* to cooked fish (not raw), and decided to undertake some bold, novel experiments in order to shed light on this mysterious idiosyncrasy. The closely observed, meticulously described account of Kustner's reaction on eating the merest trace of marine or freshwater fish is as good an account of the symptoms of food allergy as any that have been written, and it is repeated it here, exactly as translated by Prausnitz, himself [2], from the original German:-

> After half an hour there is itching of the scalp, neck and lower abdomen, with a dry sensation in the throat; soon afterwards, there is swelling and congestion of the conjunctivae, severe congestion and secretion of the respiratory mucous membranes, intense fits of sneezing, irritating cough, hoarseness merging into aphonia and marked inspiratory dyspnoea. The skin of the entire body, especially the face, becomes highly hyperaemic, and all over the body there are numerous very itching wheals, 1–2cm large, which show a marked tendency to confluence. Increased

> perspiration has not been noted. After about 2 hours, heavy salivation starts and is followed by vomiting, after which the symptoms gradually fade away. Temperature, cardiac and renal functions have always been normal. After 10 or twelve hours, all the symptoms have disappeared; only a feeling of debility persists for a day or so. After each attack, there is a period of oliguria and constipation; this may be due to dehydration and vomiting, but perhaps it is better explained by retention of water similar to what occurs in serum sickness.

Despite this dramatic sequence of events, they concluded that, although they could not identify the cause (there were no detectable *precipitins nor complement-binding and neutralising antibodies*), the effect could be transferred with the serum. This meant that when a little of Kustner's serum was injected into the skin of Prausnitz, a typical wheal and erythraema reaction occurred at the injection site, when appropriate allergen was locally administered 24 hours later. To their surprise, this local hypersensitivity persisted for more than four weeks, leading to the conclusion that the transferable serum factor was binding to the cells in the injection site. They were, of course, transferring without knowing it, human IgE.

At last, Prausnitz and Kustner were beginning to shed light on this mystery for, even though they had not demonstrated the nature of the allergy-causing agent, the effect bore all the hallmarks of an antibody, as shown in other antibody-dependent passive transfer experiments known at the time. The name of Prausnitz continued to be associated with allergy research and diagnosis for many decades, partly because the Prausnitz–Kustner test (or P–K test), derived from this classic experiment, became a standard investigative tool, until it was appreciated that the risk of transferring hepatitis with the test serum was too great to justify its use. But there was another way in which Prausnitz came to exercise a benign and helpful influence on the international allergy research community, which adds a fascinating footnote to the history of allergy research. During the 1920s and 1930s he enjoyed a sparkling academic career in Germany, holding the Chair of Hygiene and Bacteriology in the University of Breslau from 1926–1933 and becoming Director of the State Institute of Hygiene. His next move was almost certain to have been to the Prime Chair of Bacteriology in Berlin but, finding it impossible to live and work under the curse of Nazism, he and his family moved to England, where he had a license to practise medicine. After just a couple of difficult years performing research on respiratory dust disease in Manchester, he chose a completely different career path and became a country GP at Ventnor on the Isle of Wight. At this point, he adopted his mother's family name and he became known locally simply as Dr C.P. Giles, earning the epitaph of *beloved physician*. He worked in this role until about 1960, and the author is privileged to have been one of his patients in childhood on the Isle of Wight. At the local level, very few of his patients knew of the academic stature of this great man in their midst, but he did sometimes attend meetings of the influential Collegium Internationale Allergologicum in the 1950s, where he was known affectionately as *Father Giles* [3]. A photograph taken at this time is shown in Fig. 1, together with a photograph taken at the height of his academic career when he was about 50. He continued to exercise a wise and helpful influence on allergy research throughout his years on the Isle of Wight, recognised by the fact that he was invited to write the Foreword of the first

**Fig. 1** Carl Prausnitz in 1926 at the age of 50 (left) and Carl Prausnitz-Giles as a country GP at Ventnor on the Isle of Wight in 1955 (right). By permission from Karger, S., Basel, A.G. & Coombs, R.R.A. (1973) *Int Arch Allergy Appl Immunol* **45**, 1–22.

edition of the hugely influential book *Clinical Aspects of Immunology* by Gell and Coombs, which appeared in 1963, a few weeks after his death. Not only had he written the Foreword, but also he had read all the proofs and made many helpful suggestions. It is fascinating to observe that the Isle of Wight has now become well known again in the area of food allergy research, with the long-term child population studies that are conducted there. Prausnitz would have been delighted by this work, and it seems fitting that it should take place in the locality he had chosen to make his home.

It might have seemed that the progress made by Prausnitz and Kustner in 1921 was well overdue, for the symptoms experienced by Kustner had first been seen by Magendie in 1839 [4], when he observed the sudden death of dogs resulting from repeated injections of egg albumin. The same basic effect was rediscovered in 1902 when two French clinicians, Portier and Richet [5] observed similar effects when trying to prepare an antitoxin against jellyfish venom from the *Portuguese man-of-war*. In model experiments with toxin from sea anemones, they found that immunised dogs developed a rapid sequence of allergic symptoms (recognisable now as anaphylaxis), when challenged several weeks later with an identical sub-lethal injection. It was these two workers who coined the term *anaphylaxis* (greek, *ana-against* and *phylaxis-protection*), and in 1913 Richet was awarded a Nobel prize for his work on this condition.

Even with these important advances in understanding through the first 21 years of the twentieth century, the pace of development was not fast. It was not until the mid-1960s that the husband and wife team of Kimishige Ishizaka and Teruko Ishizaka [6] in the USA and Johanssen & Bennich [7] in Sweden would identify this elusive class of antibody, which became known as IgE (deriving the *E* from erythaema). Subsequently,

it became possible to work out the immunological mechanisms and pharmacological pathways involved in human allergy, leading to our present awareness of the way in which IgE antibodies bind tightly to basophils and mast cells, where they in turn bind their specific allergens (Fig. 2). When the allergen is multivalent, it binds simultaneously to several IgE molecules, thus cross-linking the antibodies involved. Such cross-linking triggers an intracellular signal cascade that causes the basophil, or mast cell, to degranulate, resulting in the release of histamine and other pharmacologically active agents that together directly cause all of Kustner's symptoms, so clearly described in 1921.

Now that we have a more complete knowledge of the fine structure and mode of action of IgE, we are still left with the deeper question of its purpose. Why has such a dangerous and apparently unhelpful mechanism evolved? What is the benefit of IgE-mediated reactions? Perhaps, the clearest answer to these questions can be seen in the role that IgE and its related accessory mechanisms (especially eosinophils, which also increase in number during allergic reactions) play in the response to helminth (worm) parasites. The exact role of IgE in the protective immune response to these types of parasite is still not fully clear, but the association between worm infections, elevated levels of IgE and dramatic increases in the eosinophil population of blood has long been known [8]. It has been shown that eosinophils can actually bind to the surface of parasites through worm-specific IgE, whereupon they degranulate and damage the target parasite through the released biochemicals [9]. Clearly, the kind of immune response involved in a Type I allergy is deeply involved in anti-parasitic reactions, and IgE working in concert with eosinophils and other factors seems to have a particular ability to take on the difficult problem of killing large parasites.

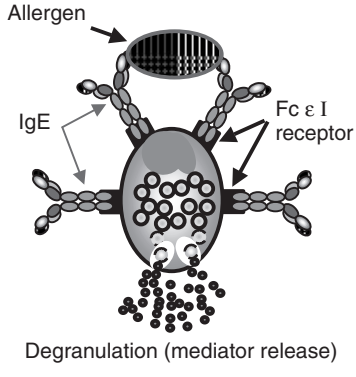


**Fig. 2** Diagrammatic representation of IgE-mediated degranulation of mast cells. Four individual IgE antibodies are depicted in a form in which each domain (about 110 amino acids) is represented by an oval. The IgE antibodies are anchored to the mast cell membrane by high affinity Fc ε I receptors. Two of the antibodies are shown binding to two different epitopes on a polyvalent allergen – a situation in which the antibodies are said to be crosslinked. This effect (crosslinking) causes a signal to be transmitted into the cell, with the result that the preformed granules (containing histamine and other pharmacologically active ingredients) are caused to expel their contents to the outside. Other (short- and longer-term) changes take place as a consequence of the allergen binding, including changes to the lipid composition of the cell membrane and the induction of certain genes.

It seems likely, therefore, that a food allergy is a misplaced anti-parasitic response, exacerbated by the solubility and rapid systemic spread of the absorbed allergen. The freedom from parasitic infection enjoyed in our modern, hygienic life style means that our elaborate anti-parasitic defences are standing by with nothing to do, and become too readily available for inappropriate engagement with innocuous dietary proteins. Perhaps, the tendency to develop allergies is an unwelcome legacy of our parasite-ridden evolutionary history. Such a view fits well with the current theory of hygiene and its unhelpful effect on the incidence of allergy in the western world. In this view, IgE is the central player in a powerful compartment of the immune system that is more adapted to primitive living than life in an advanced, modern society. Almost certainly, however, this is too simple a view and many other activities of IgE are being discovered, all of which lock it into the overall, integrated immune system. Its special functions are turning out to be essential to the normal functioning of the system as a whole.

Whatever the raison d'etre of IgE, we have to come to terms with it – at a personal level for allergic individuals – at a society level in support of a compromised group of people and at a professional level for the medical and research communities, as we search for greater understanding and practical solutions. In addition, the food industry and catering services have to understand and implement policies and procedures that safeguard allergic consumers, especially through labelling and rigorous standards in manufacture and distribution. All of this carries a major cost, but the cost of doing nothing would be far greater.

But, for the future, the most urgent need, surely, is to invest in research, so that new ways to reduce allergenicity can be discovered and new therapies can be put in place. Already, there are promising new possibilities in the form of peptide vaccines, tolerogenic routes via oral vaccination and other forms of immunomodulation. Perhaps, there will be fresh hope for sufferers of food allergy early in this new century, but it has to be recognised that, so far, the vastly improved understanding of the molecular and cellular mechanisms underlying allergy gained through the last century has done little to improve their quality of life.

The understanding gained through the PROTALL programme is an important further step forward. It is only through taking such scientific steps that we shall be able to make substantial progress, building on the past, and preparing the way with new knowledge and shared insights. Perhaps we shall at last be able to rid mankind of this poisoned inheritance, and use our knowledge to command and control our errant immune systems.

## References

1. Prausnitz, C. & Kustner, H. (1921) *Centralbl Bakteriol Abt Orig* **86**, 160.
2. Prausnitz, C. (1968) APPENDIX B Studies on Supersensitivity. In: *Clinical aspects of Immunology*, Second edition, Gell, P.G.H. & Coombs, R.R.A. (eds). Blackwell Scientific Publications, Oxford, p. 1298.
3. Coombs, R.R.A. (1973) *Int Arch Allerg* **45**, 1.
4. Magendie (1839) Vorlesungen uber das Blut, Krupp, Leipzig, cited by J. Morgenroth (1906). In: *Ehrlich's Collected Studies in Immunity*. Wiley, New York, p. 332.

5. Portier, P. & Richet, C. (1902) *C. R. Seances Soc Biol Ses Fils* **54**, 170.
6. Ishizaka, K. & Ishizaka, T. (1966) *J Allergy* **37**, 169.
7. Johanssen, S.G.O. & Bennich, H. (1967) *Immunology* **13**, 381.
8. Soulsby, E.J.L. (ed.) (1987) *Immune Responses in Parasitic Infections: Immunology, Immunopathology and Immunoprophylaxis*, Vol. 1. Nematodes, CRC Press, Boca Raton.
9. Capron, A., Dessaint, J.P., Capron, M. & Pierce, R.J. (1992) *Immunol Invest* **21**, 409.

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# 1 Food Allergies: Clinical and Psychosocial Perspectives

MONTSERRAT FERNÁNDEZ-RIVAS and SUSAN MILES

## 1.1 Definitions and classification

The lack of universal agreement on definitions and diagnostic criteria has made food allergy one of the most controversial and difficult areas among allergic diseases. The term *adverse reaction to food* applies to any clinically abnormal response induced by the ingestion, contact or inhalation of a food (or a food additive). It comprises a wide spectrum of clinical entities with different pathomechanisms, diagnostic procedures and therapeutic options. The first attempt to standardise definitions came in 1984 from the American Academy of Allergy and Immunology, and the National Institute of Allergy and Infectious Diseases [1]. Adverse food reactions were classified into *food allergy (hypersensitivity)* and *food intolerance*, depending on the existence or not of an underlying immunological mechanism, respectively. In 1995, the European Academy of Allergology and Clinical Immunology (EAACI) subcommittee on adverse reactions to food proposed a slightly modified classification based on mechanisms, which is shown in Fig. 1.1 and discussed below [2].

Adverse reactions to food can be divided into toxic and non-toxic reactions depending on whether the abnormal clinical response relies upon the food itself or upon the individual susceptibility to a certain food, respectively. Toxic reactions will occur in any exposed individual provided that the dose is high enough. Non-toxic adverse reactions are either immune mediated or non-immune mediated. The term *food allergy* refers specifically to the immune-mediated adverse reactions, whereas the term *food intolerance* should be used only in non-immune-mediated reactions [2]. Recently, the EAACI has proposed replacing the term food intolerance by *non-allergic food hypersensitivity* [3].

Food allergy can be further divided into IgE- and non-IgE-mediated reactions. Food allergens are therefore the antigenic molecules which induce the immunologic response. The role of type I, IgE-mediated reactions in food allergy has been well established and proven to be causative of clinical symptoms by double-blind,

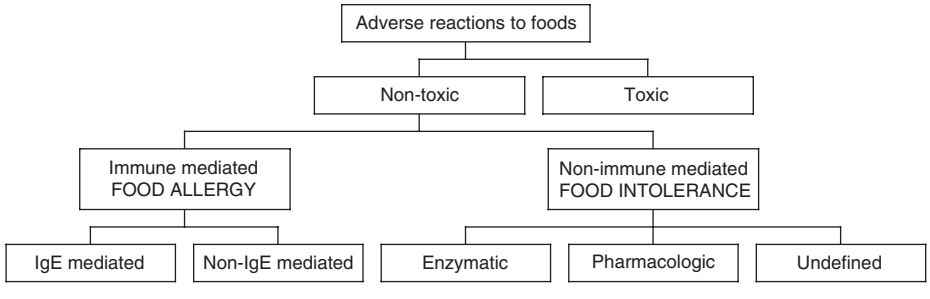


**Fig. 1.1** Classification of adverse reactions to foods.

placebo-controlled food challenge (DBPCFC). Other immune-mediated mechanisms incriminated in food allergy may play a role but they have not yet been conclusively proven [2].

Food intolerance is divided into enzymatic, pharmacologic and undefined. Enzymatic food intolerance is due to deficiencies of enzymes involved in food metabolism. The most common is secondary lactase deficiency which affects most of the adult world population. Pharmacologic food intolerance is due to substances normally present in some foods, such as the vasoactive amines, histamine and tiramin, to which some individuals are abnormally reactive [2].

This chapter will deal with IgE-mediated food allergic reactions. Most of them are induced by the ingestion of the food, but they can also be elicited through skin contact or inhalation.

## 1.2 Epidemiology

The general population perceives food allergy as a major health problem, although only a minority of the claims can be confirmed by controlled oral challenges. Recent population surveys have provided some insight into the prevalence of food allergy. It is generally accepted that food allergy affects 1–2% of the general population, and up to 8% of children less than three years of age [4–12].

The prevalence of self-reported food allergy and intolerance was studied by means of a standardised questionnaire in 17 280 adults from 15 countries who took part in the European Community Respiratory Health Survey (ECRHS) [4]. Twelve per cent of respondents reported food allergy/intolerance; however, significant differences were observed among the countries, with a range of 4.6% in Spain to 19.1% in Australia. A population study conducted in France has estimated a prevalence of food allergy of 3.2% [5]. In three consecutive surveys of 5000 American households, 14–16.6% of the respondents reported that at least one family member had a food allergy [6]. In a survey of 7500 households in the UK, approximately 20% reported an adverse food reaction. A subset of those reporting symptoms agreed to a DBPCFC study to confirm their complaints: 19.4% had a positive response, suggesting an overall prevalence of 1.4–1.8% [7]. In the Dutch adult population, self-reported food allergy and intolerance was found to be 12.4%, whereas its true prevalence established by DBPCFCs was 2.4% [8].

The prevalence of food allergy is higher in children, especially in the first three years of life. In a prospective study of American children of up to three years, 28% had experienced at least one adverse reaction to food, but the reaction was confirmed by oral challenges in only 8% [9]. Similar results were found in Finnish children aged one to six years: 23% reported adverse reactions to food, but this was confirmed in 8% [10]. In contrast, in a recent study performed in children aged 18 months from Iceland and Sweden, the prevalence of food allergy confirmed by DBPCFC was 2% [11]. In a study conducted among infants and children up to two years in Israel, the prevalence of IgE-mediated food allergic reactions was estimated to be 1.2% [12].

In summary, perceived adverse food reactions overestimate true food allergy, both in children and in the general population. The wide variable prevalences of food

allergy across the studies may be partly explained by differences in the study design and diagnostic criteria. The contribution of population-related factors such as genetic background, cultural and dietary habits, exposure to highly allergenic foods early in life, deserves further epidemiological studies.

The prevalence of food allergy is higher in patients with atopic diseases, particularly in children with atopic dermatitis. Approximately 40% of children with mild-to-moderate atopic dermatitis have a food allergy [13]. Atopy is an important predisposing factor in those food allergies arising in early infancy. Indeed, food allergy seems to start the *allergic march* from atopic dermatitis to allergic rhinitis and asthma. This is supported by the finding that food allergy in infancy, particularly egg allergy, is associated with increased aeroallergen sensitisation and increased allergic rhinitis and asthma in childhood [14, 15].

The prevalence of specific food allergies has been investigated in a number of cross-sectional studies conducted in children and adults from different countries, some of which are presented in Table 1.1 [5, 7, 8, 10–13, 16–23]. The most prevalent food allergens are milk, egg, peanut and tree nuts, fish, shellfish, soy, wheat, fruits and legumes. The relative importance of these foods varies widely with the age of the patients and the geographical location. Cow's milk and egg are the most prevalent food allergies in infants and children in all series. This reflects the worldwide consumption of these foods in this age group. Peanut allergy is frequently observed in early childhood in the USA where peanut butter is widely consumed. Allergy to fish is common in countries such as Spain [18, 22] and Japan [23] with a high consumption of fish. In Spain, fish is introduced in the child's diet around the age of 12 months, and the onset of fish allergy is predominantly observed before the second birthday [18, 22]. These studies suggest that, when the sensitisation is produced through the oral route, the timing of exposure and the dietary habits play a significant role in determining specific food allergies.

The overall prevalence of food allergy and the prevalence of allergies to specific food changes from children to adults due (in part) to the development of oral tolerance for some foods after an elimination diet. In a prospective study conducted in Danish infants, the prevalence of cow's milk allergy in the first year was around 3%, but nearly 85% lost their reactivity by the age of three years [24]. Egg allergy is often lost over time, as is fish allergy although to a lesser extent. However, tolerance to peanut, nuts and shellfish is infrequently developed, and these food allergies are generally considered life long [13, 19, 25, 26]. Food allergies may disappear in adults after an elimination diet [27], but the natural history of food allergies with onset in adulthood has been poorly investigated.

Food allergies arising in infants and small children are strikingly different from food allergies arising in adults, which may appear at any age. Food allergies in infancy must be regarded as part of the atopic syndrome. The sensitisation occurs in the gastrointestinal tract, and the allergens involved generally resist the digestion process. Typical examples are cow's milk, egg and legume allergies. Oral tolerance can develop after avoidance [9, 13, 19, 24, 25]. In contrast, most food allergies with onset in the adult age are linked to inhalant allergies, and they develop as a consequence of an IgE sensitisation to the aeroallergen which cross-reacts with the food in question. Several syndromes have been described in relation to birch, mugwort and ragweed

**Table 1.1** Prevalence of specific food allergies in relation to age and geographical location

| France | | | | Israel | | | | Spain | | | | USA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0–15 years | | 0–60 years | | Children | | >10 years | | <5 years | | >5 years | | Children (*) | | Adults | |
| Rancé *et al.* [16] | | Kanny *et al.* [5] | | Dalal *et al.* [12] | | Kivity *et al.* [17] | | Alergológica [18] | | Alergológica [18] | | Sampson [13] | | Atkins *et al.* [20] | |
| Egg | 53 | Rosaceae fruits | 14 | Egg | 42 | Peach | 75 | Egg | 44 | Fruits | 37 | Egg | 57 | Crustaceans | 50 |
| Peanut | 35 | Vegetables and legumes | 9 | Milk 7 | 28 | Almond | 39 | Milk | 44 | Nuts | 36 | Milk | 38 | Peanut | 20 |
| Milk | 13 | Milk | 8 | Sesame | 16 | Sunflower | 35 | Fish | 14 | Fish | 12 | Peanut | 29 | Egg | 10 |
| Mustard | 9 | Crustaceans | 8 | Peanut | 4 | Peanut | 31 | Fruits | 11 | Shellfish | 12 | Soy | 16 | Beer | 10 |
| Cod | 6 | Shellfish | 7 | Soy | 3 | Orange | 9 | Nuts | 7 | Egg | 10 | Wheat | 11 | Carrot | 10 |
| Hazelnut | 3 | Latex-related fruits | 5 | Tree nuts | 2 | Carrot | 6 | Legumes | 7 | Cereals | 8 | | | | |
| Kiwi | 2 | Egg | 4 | Strawberry | 2 | Tomato | 3 | | | Legumes | 6 | | | | |
| Wheat | 2 | Tree nuts | 3 | Beef | 2 | Wheat | 3 | | | Milk | 5 | | | | |
| Shrimp | 2 | Peanut | 1 | Fish | 1 | Apple | 3 | | | Vegetables | 5 | | | | |

Prevalence is given in percentage.
(*) Children with atopic dermatitis and food allergy.

pollen allergies, in mite allergic asthmatics, and in patients with respiratory allergies to bird-derived antigens (bird–egg syndrome). The foods linked to pollen and latex allergies are of plant origin, mainly fresh fruits, tree nuts and vegetables, while shellfish is associated with respiratory mite allergy [28–37]. Since pollinosis is the most frequent allergy in adulthood, and around 40% of pollen allergic patients present with an associated plant food allergy (up to 70% in birch pollinosis), vegetable foods are the most prevalent food allergens in the adult population [29, 38, 39]. The natural history of the food allergies linked to these cross-reactive syndromes is unknown. As the sensitisation to the food allergen is not produced through the oral route, the degree of compliance with the allergen elimination diet would not have an influence in the likelihood of losing the clinical reactivity in this type of food allergy.

## 1.3 Clinical manifestations

IgE-mediated food allergic reactions to ingested food may involve one or more target organs, including the skin, the gastrointestinal and respiratory tracts, and the cardiovascular system. The clinical manifestations linked to immediate food allergic reactions reported in controlled blinded challenges are listed in Table 1.2 [19, 21, 40].

### 1.3.1 *Generalised reactions*

*Anaphylaxis* is the severest manifestation of food allergy. It is a generalised allergic reaction that may involve multiple organ systems. It generally occurs within minutes of food ingestion, and the patients may develop pruritus, urticaria, angioedema, laryngeal edema, bronchospasm, abdominal cramps, vomiting, diarrhoea, cardiac arrhythmias, hypotension and shock [26, 36, 41–49]. In the so-called *exercise-induced anaphylaxis*, the intake of a specific food or (more rarely) of any food, induces a generalised reaction only if the patient exercises in the 2–4 hours following the ingestion [50].

**Table 1.2** Clinical manifestations of IgE-mediated reactions to foods reported in blinded challenges

| |
|---|
| **Generalised reactions** |
| Anaphylaxis |
| Food-dependent, exercise-induced anaphylaxis |
| **Cutaneous reactions** |
| Acute urticaria/angioedema |
| Food-dependent, exercise-induced urticaria/angioedema |
| Atopic dermatitis |
| **Gastrointestinal reactions** |
| Oral allergy syndrome |
| Gastrointestinal anaphylaxis |
| **Respiratory reactions** |
| Rhinoconjunctivitis |
| Laryngeal edema |
| Asthma |

Food allergies are the most frequent cause of systemic anaphylaxis seen at emergency rooms, accounting for at least one-third of the cases. The foods most commonly involved are peanut, tree nuts, fresh fruits, celery, seeds, legumes, seafood, egg and milk. Fatal food anaphylaxis is more frequently observed among adults and adolescents [36, 41–49].

### 1.3.2 *Cutaneous reactions*

Skin symptoms are the most common manifestations of immediate food allergy [19, 21, 26, 36, 40, 49]. Acute *generalised urticaria and/or angioedema* are frequently seen in combination with symptoms of other target organs, but may be present as the sole manifestation. Sometimes only a generalised erithema, most often pruritic, is observed. *Food-dependent exercise-induced urticaria/angioedema* has also been described. Food allergy is exceptionally involved in chronic urticaria.

The role of food allergy in *atopic dermatitis* has been investigated mainly in children. It has been demonstrated that food allergy is a pathogenic factor in up to 40% of infants and young children with moderate-to-severe atopic dermatitis. Studies conducted in American children have shown that the foods most frequently involved are egg (57%), milk (38%), peanut (29%), soy (16%) and wheat (11%). When these children were placed on an appropriate allergen elimination diet they experienced a marked improvement of their dermatitis. One-third of these symptomatic food allergies are outgrown in two to three years. The probability of developing tolerance depends on the food allergen (frequent to soy, rare to peanut) and on the compliance to the avoidance diet [13, 19, 26, 51].

The role of food allergy in atopic dermatitis seen in adolescents and adults is not elucidated. Studies in adults with severe atopic dermatitis are scarce, but they have not shown a significant role for food allergy, or success in clearing of skin lesions during elimination diets [52, 53].

### 1.3.3 *Gastrointestinal reactions*

*Oral allergy syndrome* (OAS) is a form of contact urticaria confined to the lips and oropharynx. Symptoms generally appear within 5 to 15 minutes following the food ingestion and consist of pruritus of the lips, tongue, palate and throat; mild angioedema at the same sites may be associated. Rapid and spontaneous resolution is seen in most cases, although some patients may develop subsequent digestive complaints (gastric pain, nausea, vomiting) and/or systemic involvement [32, 54]. The clinical presentation seems to be related to the stability to the digestion process of the allergens involved [28, 49, 55–58].

OAS is most often seen in adolescent and adult patients allergic to pollens of birch, ragweed, mugwort and grasses. The foods most frequently involved are of plant origin, generally fresh fruits and vegetables. The basis for these associations is the existence of cross-reactivity between the pollen allergens and the linked vegetable foods [28–32, 35, 37, 55, 59–66]. Due to the high prevalence of pollinosis in the adult population, and its frequent association with plant food allergies, OAS is the most frequent clinical presentation of food allergy seen in adult patients [29, 38, 39].

Although epidemiological studies are lacking, OAS appears to have become more prevalent in the past decades. This can be related to the increase in prevalence of pollen allergies in western countries [38], and to an increased awareness of this clinical presentation of food allergy.

Food allergic reactions at the gastrointestinal tract (also called gastrointestinal anaphylaxis) induce symptoms such as nausea, vomiting, abdominal pain and diarrhoea. They often accompany allergic manifestations in other target organs, but sometimes may be the only symptoms noted [19, 21, 40].

### 1.3.4 *Respiratory reactions*

*Rhinoconjunctivitis, asthma* and *laryngeal edema* have been observed during controlled oral food challenges. It has been documented that food-induced allergic reactions may induce acute bronchospasm and an increase in bronchial hyperreactivity [19, 67, 68]. However, these respiratory symptoms are exceptionally seen as the sole manifestation of food allergy. They are most often seen in patients with atopic dermatitis, food allergy and associated rhinitis and/or asthma, or in combination with acute skin or digestive symptoms, generally in the context of an anaphylaxis. Acute asthma attacks observed in systemic anaphylaxis may be extremely severe, and are the most frequent cause of death in the reported cases of fatal food-induced anaphylaxis [41–44, 48].

Some patients allergic to fish, crustaceans and legumes (with reactions after ingestion) may suffer from rhinitis and asthma induced by the inhalation of the steam from cooking these foods [69–72].

### 1.3.5 *Occupational rhinitis and asthma induced by food inhalation*

IgE sensitisation to foods may be induced by the inhalation of airborne food allergens in the occupational setting. Many foods of animal and plant origin, such as crustaceans, molluscs, fish, egg, poultry, cereal flours, soybeans, castor and coffee beans, spices, plant enzymes such as bromelain and papain, and fresh vegetables, are known to induce occupational asthma and rhinitis. Their high molecular-weight allergens can be dispersed as dust (cereals flours in baker's asthma), aerosol (fresh vegetables in homemaker's asthma) or vapour (crustaceans in the seafood processing industries). Farmers, workers involved in food processing, manufacturing, transportation, trade or retailing, cooks, and even housewives, are the population at risk. Epidemiological data in this farm and food sector are scanty. It is known that occupational asthma occurs in 3% to 10% of workers exposed to green coffee beans, in 15% of snow crab processing workers and in 10% to 30% of bakers. These patients frequently present an associated food-induced contact urticaria or hand dermatitis [72–80].

### 1.3.6 *Contact skin reactions induced by foods*

Contact skin reactions induced by foods through an IgE-mediated mechanism may present as contact urticaria and protein contact dermatitis. Contact urticaria refers to an immediate wheal and flare response after cutaneous exposure of intact skin.

Protein contact dermatitis refers to the chronic, generally hand, dermatitis of food handlers, who experience immediate reactions when the affected skin is exposed to certain foods. Workers of food processing industries, food handlers, cooks, housewives, farm workers, are at the risk groups. The foods most frequently involved are raw fish, shellfish, vegetables and fruits. Patients become sensitised by cutaneous exposure, and some of them may also present an associated food-induced respiratory allergy [81–83].

Most of the (raw) foods to which the patient is sensitised through inhalation or skin contact do not elicit symptoms when ingested. This can be partly explained by the modifications of the food allergens induced by cooking and digestion [76, 79–81, 83].

## 1.4 Diagnosis

The diagnostic approach to allergic food reactions comprises three steps. The first step includes the medical history and physical examination. On the basis of symptoms and timing of the reaction, the physician attempts to identify the suspected food and to determine whether the reaction is likely to involve an immunologic mechanism. This first step is absolutely necessary to decide on the subsequent diagnostic tests to be performed. The second step includes skin tests and *in vitro* assays, which can confirm a sensitisation to the food. However, for the conclusive diagnosis of a food allergy it is necessary to demonstrate, in a third step, by an oral challenge, that the food to which a sensitisation has been found is responsible for the patient's symptoms.

### 1.4.1 *Skin and* in vitro *tests for determination of specific IgE to foods*

Skin prick tests (SPTs) are most frequently used as the first test to screen for specific IgE to foods. SPTs are not uncomfortable for the patient, they are easily performed, quick (the result is available in 15 minutes), safe and cheap. For these reasons they are the method of choice to demonstrate an IgE response to foods. *In vitro* tests are recommended in patients with extensive skin disease, dermographism, who cannot discontinue antihistamines, or with a history of an extreme sensitivity [2, 25, 84, 85].

The diagnostic accuracy of skin and *in vitro* tests depends on the quality of the food allergen extracts. In contrast to aeroallergens, food allergen extracts have not been standardised [86–91]. The performance characteristics of tests for egg, milk, peanut, fish, wheat and soy have been extensively studied, particularly in children with atopic dermatitis. SPTs and CAP to egg, milk, peanut and fish are comparable, with excellent sensitivity and negative predictive accuracy (most >90%), but poor specificity and positive predictive accuracy (50–85%). Therefore, a negative test with these food extracts is a good method to rule out an IgE-mediated food allergy. In contrast, a positive test is only suggestive of the presence of a clinically relevant food allergy, and the final diagnosis should rely on an oral challenge [86–90]. However, a positive test in a patient who has experienced a systemic reaction after the ingestion of an isolated food should be considered diagnostic [25]. By means of the Pharmacia CAP system, cut-off points with a 95% positive predictive value have been

established for egg, peanut, milk and fish. The application of these cut-off points in clinical decisions can reduce the need to perform DBPCFCs in a significant number of patients [88, 89].

The diagnostic accuracy of SPTs and *in vitro* IgE assays for fresh fruits and vegetables is poor. The sensitivity of the tests is generally low, as it is shown in Table 1.3 for the Rosaceae fruits, presumably due to the lability of the allergens involved [32, 91–93]. To overcome this problem the prick–prick test has gained popularity. In this test the lancet is plunged several times into the food immediately before pricking the patient's skin with it [94]. Nowadays, the prick–prick test is the most sensitive test with fresh foods [91]. It is also useful when there are discrepancies between a suggestive medical history and a negative SPT with a commercial extract, or when a specific food extract is not available. The inconveniences of the prick–prick test are the impossibility of standardisation and the dependence on the availability of the fresh food.

Positive SPTs and serum-specific IgE to fruits and vegetables are commonly seen in tolerant patients. These false positive results are an expression of IgE cross-reactivity. This is frequently found in pollen allergic patients sensitised to the major birch pollen allergen (Bet v 1), to profilin or to carbohydrate determinants of glycoproteins [28–35, 37, 55, 59–66]. This latter cross-reactive structure seems to hamper exclusively the specificity of the *in vitro* assays [95, 96]. To overcome the poor specificity of tests, clinicians have to confirm clinical reactivity or tolerance by means of controlled oral challenges.

In recent years purified, natural and recombinant major plant food allergens have been applied in diagnosis, with the aim of improving sensitivity, specificity and reproducibility of *in vivo* and *in vitro* tests. The sensitivity of tests performed with major allergens of Rosaceae fruits is presented in Table 1.3 [97–101]. Available data of tests performed with recombinant allergens from plant foods such as peanut, celery, hazelnut, apple, peach, pear and cherry show sensitivities generally higher than 80%. The combination of several recombinant allergens from the same food increases the sensitivity [97–102].

### 1.4.2 *Food challenges*

SPTs and *in vitro* IgE assays are useful methods to demonstrate the presence of food specific IgE antibodies, but they do not establish the diagnosis of clinical food allergy. The oral challenge is the diagnostic test which provides conclusive evidence of a food allergy [2, 20, 21, 86, 88–92].

Oral food challenges should always be performed after an elimination diet of the suspected allergens. The resolution of symptoms during the elimination diet suggests a correct clinical suspicion, which has to be confirmed by an oral provocation. Food challenges are performed to confirm the diagnosis and recommend a correct elimination diet. However, in those patients presenting a severe systemic reaction after the ingestion of an isolated food to which specific IgE is demonstrated, food challenges are not needed to confirm the diagnosis [25]. As oral tolerance may develop after a correct elimination diet, particularly in children, longitudinal provocations should be performed to see if the problem is resolving.

**Table 1.3** Sensitivity of skin and *in vitro* IgE assays to Rosaceae fruits

| Authors | Fruit | Prick–prick test Fresh fruit | SPT extract | Serum IgE determination | Purified allergens | | |
|---|---|---|---|---|---|---|---|
| | | | | | Allergen | SPT | Serum IgE determination |
| Ortolani *et al.* [32] | Peach | 86 | 11 | 59 | | | |
| | Apple | 84 | 4 | 70 | | | |
| Ortolani *et al.* [91] | Peach | 59 | 14 | – | | | |
| | Apple | 81 | 2 | 70 | | | |
| | Pear | 43 | 0 | – | | | |
| Cuesta-Herranz *et al.* [92] | Peach | 100 (*) | 4 | | | | |
| | | | 5 | | | | |
| | | | 13 | | | | |
| | | | 74 | | | | |
| Rodríguez *et al.* [93] | Peach | 71 | | 68 | | | |
| | Apple | 53 | | 65 | | | |
| | Pear | 53 | | 29 | | | |
| | Apricot | 41 | | 53 | | | |
| | Plum | 56 | | 47 | | | |
| | Strawberry | 38 | | 41 | | | |
| García-Sellés *et al.* [97] | Peach | 96 | 96 | 100 | Natural Pru p 3 | 91 | |
| Son *et al.* [98] | Apple | | | | rMal d 1 | | 100 |
| Karamloo *et al.* [99] | Pear | | | | rPyr c 1 | | 100 |
| Scheurer *et al.* [100] | Cherry | | | | rPru av 1 | | 96 (G) |
| | | | | | rLTP | | 3 (G), 100 (I) |
| Ballmer-Weber *et al.* [101] | Cherry | | | | rPru av 1 | 92 (Sw), 0(S) | |
| | | | | | rPru av 3 | 4 (Sw), 89(S) | |
| | | | | | rPru av 4 | 17 (Sw), 22(S) | |

Sensitivity is given in percentage. SPT: skin prick test. (*) A positive prick–prick test to fresh peach was the inclusion criterium. LTP: lipid transfer protein. (G) German, (I) Italian, (Sw) Swiss and (S) Spanish patients.

Oral food challenges may be performed openly (patient and physician are aware of the food ingested), single-blind (only the physician knows the content of the challenge) or double-blind (neither the patient nor the physician is aware of the content of the challenge). The blind challenges may be placebo-controlled. The DBPCFC is considered the *gold standard* for the diagnosis of food allergy, although the need to employ it routinely in the clinical setting continues to be debated. DBPCFCs are required for research studies, chronic disorders such as atopic dermatitis, patients who appear to have multiple food allergies, and when the patient's subjective complaints may bias accurate symptom assessment. The characteristics, advantages and disadvantages of the various provocation procedures are presented in Table 1.4 [90, 103, 104].

Food challenges should be performed in the hospital where emergency care is immediately available. The food is administered to the patient in fasting conditions, starting with a dose unlikely to provoke symptoms, according to the eliciting dose reported in the medical history or in the last positive provocation test. Incremental amounts of food (double) are given at time intervals slightly longer than expected to produce symptoms, until a positive reaction appears or the patient eats a normal amount of the food. For blind tests, foods can be given in capsules (dehydrated) or in a vehicle which should mask taste, consistency, colour and odour. All negative blind challenges must be confirmed by an open feeding with normally processed food [90, 103–106].

Open food challenges (OFC) are useful to reintroduce a food in the regular diet after a lack of response to the elimination diet, and when the SPT is negative, the history is not suggestive, and the patient has been avoiding the food [90]. OFCs may be useful as a first step of the provocation procedures. If the OFC is negative, the food is introduced in the diet. Whenever the OFC is positive, it is recommended to perform a DBPCFC [2, 90].

**Table 1.4** Oral challenge procedures

| Method | Characteristics | Advantages | Disadvantages |
|---|---|---|---|
| DBPCFC with capsules | Dehydrated food<br>Can be titrated | Good blinding<br>Suitable for additives | Limitations with high number of capsules<br>OAS is not diagnosed<br>Possible modifications of food allergenicity (labile allergens) |
| DBPCFC with foods | Fresh food<br>Can be titrated | Suitable for OAS<br>Normal route of administration | Laborious, resource demanding |
| OFC with foods | Fresh food<br>Can be titrated | Reproduces normal feeding<br>Easily performed<br>Performed after a negative DBPCFC confirms oral tolerance in normal feeding<br>Gives confidence to the patient upon food tolerance<br>If negative, DBPCFC is not needed | Risk of false positive results |

DBPCFC: double-blind, placebo-controlled food challenge; OFC: open food challenge; OAS: oral allergy syndrome.

## 1.5 Treatment

The only proven therapy in food allergy is the strict avoidance of the offending food. Elimination diets, particularly if a large number of foods are involved, may lead to eating disorders and malnutrition. Dietician supervision may be necessary, particularly in growing children. In order to avoid accidental exposure to a hidden allergen in a processed food, patients (or their parents) should check the labels and should be aware of the different names the same food can be given in ingredient lists [107–109]. Clinical reactivity to a food is generally very specific and patients are not generally allergic to more than one member of a botanical family or animal species [93, 108, 109]. However, if the tolerance of closely related cross-reactive foods has not been assessed after a confirmed diagnosis to a member of the family, the patient should be instructed on the possible danger related to cross-reactivities. Meals at school, restaurants or friend's homes may be dangerous. As will be discussed below, elimination diets require constant vigilance and may restrict social activities. Psychological support for the patients and their families should be considered. Associations of food allergic patients provide extensive information and social support, and may be of great help [107, 110, 111].

Given the difficulty of avoiding food allergens, patients are at risk of experiencing allergic reactions due to accidental food ingestion. All the patients should be trained in the early recognition and treatment of reactions, and given rescue medication. If they are at risk for severe anaphylaxis they should be instructed on the self-injection of adrenaline and carry it. School staff should be informed of the children's allergies, and know how to treat a reaction [107, 110, 111].

## 1.6 The impact of food allergies on quality of life

The impact of allergic diseases is dissimilar to many other diseases as, except for a small number of people, allergic diseases are rarely fatal. Instead they adversely affect quality of life for a prolonged period. Quality of life encompasses factors such as health, financial security, standard of living, family and friends, and spiritual contentment [112, 113]. Health-related quality of life (HRQL) is the individual's perception of the effects of an illness, and its treatment, on him/herself. It includes aspects of physical, psychological and social well-being [114].

Studies in the UK, examining admissions and discharges with anaphylaxis as the primary diagnosis, have indicated that there has been an increase in anaphylaxis in the last decade [115, 116]. Around a third of anaphylactic cases are food-induced. It should be noted that many cases of anaphylaxis are likely to be managed in accident and emergency departments, without resulting in admission; as a result these studies may represent a substantial underestimate. Somewhat reassuringly, Macdougall *et al.* [48] found that, in children, the risk of death is small (approximately 1 in 800 000 per year). The risk was greater in the 10–15-year age group than in children under ten years. It was also greater for allergic individuals experiencing both food allergy and asthma, than for just food-allergic individuals. Prevention of food-induced

anaphylaxis requires appropriate identification and complete dietary avoidance of the specific food allergen [43].

### 1.6.1 *Vigilance and stress*

Food allergies, particularly in children require constant vigilance, which can be a source of stress [117]. For example, allergic individuals (and their families) need to recognise the signs of inadvertent ingestion, including anaphylaxis, and they may need to learn how to provide emergency treatment. Parents of food-allergic children need to monitor their child's diet and behaviour more closely than parents of non-allergic children. Furthermore, labels need to be checked all the time in case manufacturers have changed the ingredients, and food-allergic individuals need to be able to recognise all forms of the allergen.

Mandell *et al.* [118] note that the parents of food allergic children alternate between a state of low anxiety and one of high anxiety. During times of high anxiety, family members report experiencing a dysfunctional level of stress and fear. However, when it is too low, family members do not necessarily exercise sufficient avoidance behaviour, or emergency preparedness. Increased anxiety can be due to circumstances such as an incident of accidental exposure, the discovery of new information about a potential risk (such as news of incorrectly labelled food) and developmental changes that potentially expose the child to increased risk (such as increased independence). During anxious periods, vigilance is also high.

Care of an allergic child can interfere with parental employment, in that employment may be reduced, ceased or not started. Also parents of allergic children may accompany their children in social situations where non-allergic children may come alone, which could be awkward for the allergic child and impact on its social development [119]. An allergic child in the family may also impact on the family relationship. For example, parents may become anxious and overprotective of the allergic child. They may even feel guilty, or hostile towards the child. Furthermore, siblings may be deprived of needed attention, which may cause resentment [113]. Allergic children may feel negatively about the restriction in their activities, such as participation in school trips [118].

Primeau *et al.* [120] assessed quality of life in the family relations of both adults and children with peanut allergy compared to those with rheumatological disease. They found that peanut allergic individuals were significantly more disrupted in their daily activities and more impaired in their family relations than those with rheumatological disease. This is supported by the findings of Sicherer *et al.* [121] who found that childhood food allergy had a significant impact on: perception of overall health and illness, distress and worry experienced by parent, limitation and interruption in usual family activities, and family tension as a result of the child's health. Food allergies in children can also lead to absence from school [122], which may impact on educational development.

The allergic individual will have considerably fewer choices than non-allergic individuals, which can also be a source of stress as well as being inconvenient. For example, they may not be able to eat out where they choose, the increasing use of precautionary labelling will limit their food choices (discussed below), and their

choice of holidays or leisure activities may also be affected. It may also have a negative impact on self-perception [123].

### 1.6.2 *Financial costs of food allergy*

There can be an impact of allergy on an individual's working life. For example, allergic conditions may lead to work absenteeism. This includes, *sick days* because of the allergic condition, family members having to take time off to care for an allergic individual, and time off because of a work-related injury related to the condition or medication used to control it [124]. Related to this, allergic individuals may suffer restricted activity days; this is where individuals are limited in their activity by their allergic condition. It is usually the result of uncontrolled or poorly controlled symptoms. Furthermore, allergic individuals may find that they are unable to work regular hours or overtime, or that they lose promotion opportunities. They may have to shift from full-time to part-time employment, or to jobs that are less physically demanding [125]. People suffering from allergic diseases may also experience a total loss of employment; due to qualifying for total work disability or from taking early retirement [125]. Such an impact on employment may have an adverse effect on the allergic individual's income.

Additional costs incurred by the allergic individual and his/her family may include the financial loss associated with early mortality, loss of education and reduced long-term career attainment [126]. Further costs may be associated with allergen avoidance measures (such as special diets or environmental adaptations), alternative medicines and requiring home help (such as child care). Most of the work investigating the economic impact of allergy has been focused on asthma and allergic rhinitis; however many of the same costs, such as those described above, will appropriate for food allergies.

### 1.6.3 *The impact of inadequate labelling*

The potential for exposure to common allergens can occur on a daily basis. As avoidance is the only effective means to prevent allergic reactions to foods, constant monitoring of food consumption is necessary. This means that allergic individuals and people who shop for them must rely on ingredient labels to select safe food. Labels must be complete and accurate [127]. Mistakes have the potential to be fatal. However, current labelling regulations do not require that every ingredient in a product be declared. For example, in Europe, the components of a compound ingredient present in the final food product at less than 25% do not need to be labelled. In the White Paper on Food Safety, the European Commission announced its intention to propose an amendment to the Labelling Directive 2000/13/EC. In particular, this amendment aims to abolish the *25% rule*. It will also establish a list of ingredients liable to cause food allergies or intolerance that will be labelled. Taylor and Hefle [127] note that whilst all of these recommendations are positive and will be helpful to food-allergic consumers, each individual country must adopt these recommended regulations before they can be enacted. At the time of writing, this amendment is under consideration.

Inadequate labelling of processed food is a major problem for food-allergic consumers, and can result in accidental ingestion of hidden allergens. One issue is that of misleading labels [128, 129]. One such labelling problem relates to ingredient switching, where manufacturers change the recipe, or switch ingredients without making this clear on the label. Such changes should be highlighted on the packaging [130]. A second problem is the use of complex or uncommon terms, or symbols to describe allergens, for example *whey* instead of *milk*. Common, easily identifiable names should be used to describe ingredients that may be allergenic [130]. The use of ambiguous terms such as *natural flavours* or *spices* is also problematic. Allergens should be declared when they are present in spices or natural flavours [129]. The IGD/PIC Food Labelling and Consumer Information Steering Group voluntary guidelines for the UK food industry recommend that common allergens should always be labelled in the ingredients list [130].

Mistakes can also arise from errors in reading the labels. Joshi *et al*. [129] investigated the accuracy with which parents who were restricting their children's diets because of food allergy were able to identify the restricted foods on food product labels. They found that the parents made numerous mistakes when reading product labels. Particularly problematic were products containing soy and milk, with 78% and 93% (respectively) unable to identify the offending allergen. Nearly half of the parents of peanut allergic children were not able to identify peanut in all the samples of food products containing peanut. The authors also found that almost half the parents had needed to contact manufacturers to assist with the interpretation of food ingredient labels prior to the study. Other authors also found evidence that warnings specifically related to nut contamination are difficult to read, due to problems such as shiny paper, small fonts, or the warnings being away from the ingredient information [131, 132].

There is also scope for accidental ingestion due to the contamination of safe food. For example, if the same serving utensil is used for different foods or if a manufacturing plant uses the same equipment to make different products without adequate cleaning. Contamination may also occur if the same oil is used to cook chips and fish, or if the same slicer is used to cut both meat and cheese at a deli counter [128].

Schäppi *et al*. [133] investigated the presence of hidden allergens in food products, and found that of 46 samples (cereals, cereal bars, cookies and various types of snacks), 19 were shown to contain undeclared peanut material. Reasons for cross-contamination of peanut-free products included: contaminated raw materials, common transport containers for peanuts and other foods, a lack of separate production lines and equipment for peanut-containing and peanut-free foods, processing of peanut-free products immediately after peanut-containing products, unsafe rework-management and insufficient cleaning steps.

The potential for accidental ingestion may be even more of a problem when eating out, where ingredient statements are not readily available, and staff lack the knowledge needed to assist the food-allergic consumer. Furthermore, there is a tendency for food-allergic individuals to self-manage their allergy when eating out, in other words, they do not tell the restaurant staff that they are allergic to a particular food, believing that they can recognise *problem* dishes [134].

Many of the most severe food-allergic reactions occur outside the home. Eigenmann and Zamora [134] found that food-induced anaphylactic reactions in adults and

children occurred at home, in restaurants, at school, at relative/friends' house, at the site of leisure activities, at work, in church, in hospital wards and in food stores. Allergic reactions at school can occur even when children are provided with food to eat by their parents and are not allowed to eat anything else, or swap food with their friends [135]. A study conducted in French schools indicated that over a third of reactions to food were due to the inadvertent ingestion of the food or of a hidden allergen [111]. For these reasons, it is critical that schools are able to recognise and treat allergic reactions, as it is clear that reactions can occur in schools even where every effort is made to minimise the risk. However, there is evidence that food allergy awareness in schools can be low. In a questionnaire study conducted in the US, Rhim and McMorris [136] found that there was a lack of structured, school-wide staff education on food allergies, there were also gaps in avoidance measures such as reading of food labels, a lack of written emergency treatment plans and a lack of immediate accessibility of emergency epinephrine. Nowak-Wegrzyn *et al.* [135] also found that the onus on food allergy education for staff fell to the parents. This can be problematic if the parents only talk to the child's teacher. Details of allergen avoidance, how to recognise inadvertent ingestion and treatment need to be widely communicated amongst the school staff.

### 1.6.4 *Precautionary labelling*

A further issue relates to anecdotal evidence of frustration with the increasing use of precautionary labelling, such as *may contain* labels [117, 132, 137]. Some food-allergic individuals believe that this labelling is over-inclusive and designed to cover liability for contamination when the risk is probably very small. Hourihane [137] notes that such precautionary labelling may appear on products that food-allergic individuals have eaten safely for many years (perhaps due to changes in manufacturing processes). On the basis of safe previous consumption, individuals may consider the risk acceptable and continue to eat the product. There is evidence that peanut-allergic consumers take longer to shop than non-allergic consumers, pay more for their food shopping, and have less choice in the food available to them [132, 138]. Such a situation can add to the inconvenience and stress already experienced by the food allergic individual.

Widespread use of precautionary labelling can be an important barrier to allergic individuals leading a normal life, particularly when such labelling is found on everyday staples. McCabe *et al.* [131] assessed three common food products (cereal, biscuits and confectionary) in four major retail supermarket stores in the UK. They inspected 630 food products and classified them according to nut risks: 15% marked as containing nuts (obvious nut content); 25% were nut-free (no trace of nuts noted on product wrapping); and 60% had warning of nut risk (possibly contaminated by nut products or produced in a unit that handled nuts). As noted by Gowland [117], these findings suggest that of a sample of 20 UK cereals, biscuits and confectionary, 3 would contain nuts deliberately, 5 would appear free and 12 would have some contamination risk. Thus, from the nut-allergic consumer's viewpoint, instead of a choice of 17 products from 20 (which did not have nuts as an ingredient) they could choose from only 5. Allergic individuals do not deserve such a restricted food choice.

Hourihane [137] notes that precautionary labelling may also have an impact on non-allergic shoppers. A common hazard control response seen in schools with a peanut-allergic child is to restrict snacks containing peanut in the school. Hourihane argues that if we assume that the average class size in Britain is 30 pupils, and if each pupil comes from a family of four members, then for each peanut-allergic child there may be 120 unaffected members of the public who may be dissuaded from buying a snack of other food because of a *may contain* label.

There is anecdotal evidence from patient groups that teenagers and young adults disregard allergen trace information [117, 132]. These allergic individuals cannot believe that such a huge proportion of products on sale may put their lives at risk. They are cynical about the reasons behind the use of precautionary labelling, and they believe that if the risk were serious, the warnings on the packet would be bigger, easier to read and find.

A further problem with precautionary labelling is that the current regulatory situation varies around the world. This can confuse food-allergic consumers as they travel or purchase imported packaged foods [127]. Additionally, an over use of *may contain* labelling may lead to a devaluation of its message. Such a situation would mean that a lot of people may be putting their health, or their lives at risk. Precautionary labelling must not be a substitute for good manufacturing practices.

### 1.6.5 *Patient groups*

Allergic individuals are becoming increasingly informed about risks and safe management of food allergy through patient groups such as the Anaphylaxis Campaign in the UK and the Food Allergy Network in the US. Such groups can provide social and informational support to allergic individuals and their families. They may provide advice as to how allergic individuals can deal with their food allergy effectively in different situations such as travelling, or sending an allergic child to school. They may have information about food alerts (foods which have been found to contain unlabelled allergens) or lists of currently *safe* foods. The support of such groups should help allergic individuals handle their food allergy safely and confidently, with less impact on their daily activities.

### 1.6.6 *Perceived food allergy or intolerance*

There is evidence that prevalence of perceived adverse reactions to food (around 20%) is considerably higher than the prevalence of actual food allergy (less than 2%) [139, 7]. Such results suggest that most self-reported illness to food is not due to food allergy. This may indicate that the number of people modifying their diet is greater than the number who need to. People suffering from perceived food allergy and intolerance alter their diet (and sometimes that of their family) of their own accord, not on the advice of a doctor or dietician [140–142]. Such dietary restrictions may have an adverse effect on nutrient intake. It is also likely to be inconvenient and expensive. Public education is required to ensure that people are not unnecessarily restricting their dietary intake.

## References


1. American Academy of Allergy and Immunology/NIAID (1984) *Adverse reactions to foods*, Anderson, J.A. & Sogn, D.D. (eds). NIH Publication 84-2442, Washington, pp. 1–6.
2. Bruijnzeel-Koomen, C., Ortolani, C., Aas, K. *et al*. (1995) Adverse reactions to food. Position paper. *Allergy* **50**, 623–635.
3. Johansson, S.G.O., O'B Hourihane, J., Bousquet, J. *et al*. (2001) Revised nomenclature for allergy. Position paper. *Allergy* **56**, 813–824.
4. Woods, R.K., Abramson, M., Bailey, M. & Walters, E.H. (2001) International prevalences of reported food allergies and intolerances. Comparisons arising from the European Community Respiratory Health Survey (ECRHS) 1991–1994. *Eur J Clin Nutr* **55**, 298–304.
5. Kanny, G., Moneret-Vautrin, D.A., Flabee, J., Beaudouin, E., Morisset, M. & Thevenin, F. (2001) Population study of food allergy in France. *J Allergy Clin Immunol* **108**, 133–140.
6. Altman, D.R. & Chiaramonte, L.T. (1996) Public perception of food allergy. *J Allergy Clin Immunol* **97**, 1247–1251.
7. Young, E., Stoneham, M.D., Petruckevitch, A., Barton, J. & Rona, R. (1994) A population study of food intolerance. *Lancet* **343**, 1127–1130.
8. Jansen, J.J.N., Kardinaal, A.F.M., Huijbers, G., Vlieg-Boerstra, B.J., Martens, B.P.M. & Ockhuizen, T. (1994) Prevalence of food allergy and intolerance in the adult Dutch population. *J Allergy Clin Immunol* **93**, 446–456.
9. Bock, S.A. (1987) Prospective appraisal of complaints of adverse reactions to foods in children during the first 3 years of life. *Pediatrics* **79**, 683–688.
10. Kayosaari, M. (1982) Food allergy in Finnish children aged 1 to 6 years. *Acta Paediatric Scand* **71**, 815–819.
11. Kristjansson, I., Ardal, B., Jonsson, J.S., Sigurdsson, J.A., Foldevi, M. & Björksten, B. (1999) Adverse reactions to food and food allergy in young children in Iceland and Sweden. *Scand J Prim Health Care* **17**, 30–34.
12. Dalal, I., Binson, I., Reifen, R. *et al*. (2002) Food allergy is a matter of geography after all: sesame as a major cause of severe IgE-mediated food allergic reactions among infants and young children in Israel. *Allergy* **57**, 362–365.
13. Sampson, H.A. (1997) Eczema and food hypersensitivity. In: *Food Allergy: Adverse Reactions to Foods and Food Additives* Metcalfe, D.D., Sampson, H.A. & Simon, R.A. (eds). Cambridge, Mass: Blackwell Science, pp. 193–209.
14. Kulig, M., Bergmann, R., Niggemann, B., Burow, G. & Wahn, U. (1998) Prediction of sensitization to inhalant allergens in childhood: evaluating family history, atopic dermatitis and sensitization to food allergens. The MAS Study Group. Multicentre Allergy Study. *Clin Exp Allergy* **28**, 1397–1403.
15. Tariq, S.M., Matthews, S.M., Hakim, E.A. & Arshad, S.H. (2000) Egg allergy in infancy predicts respiratory allergic disease by 4 years of age. *Pediatr Allergy Immunol* **11**, 162–167.
16. Rancé, F., Kanny, G., Dutau, G. & Moneret-Vautrin, D.A. (1999) Food hypersensitivity in children: clinical aspects and distribution of allergens. *Pediatr Allergy Immunol* **10**, 33–38.
17. Kivity, S., Dunner, K. & Marian, Y. (1994) The pattern of food hypersensitivity in patients with onset after 10 years of age. *Clin Exp Allergy* **24**, 19–22.
18. Alergia a Alimentos. (1995) In: *Alergológica. Factores Epidemiológicos, Clínicos y Socioeconómicos de las Enfermedades Alérgicas en España. Sociedad Española de Alergología e Inmunología Clínica y Alergia e Inmunología Abelló, S.A*., Madrid, pp. 163–183.
19. Sampson, H.A. (1999) Food allergy. Part 1. Immunopathogenesis and clinical disorders. *J Allergy Clin Immunol* **103**, 717–728.
20. Atkins, F.M., Steinberg, S.S. & Metcalfe, D.D. (1985) Evaluation of immediate adverse reactions to foods in adults. I. Correlation of demographic, laboratory and prick skin test data with response to controlled oral food challenge. *J Allergy Clin Immunol* **75**, 348–355.
21. Atkins, F.M., Steinberg, S.S. & Metcalfe, D.D. (1985) Evaluation of immediate adverse reactions to foods in adults. II. A detailed analysis of reaction patterns during oral food challenge. *J Allergy Clin Immunol* **75**, 356–363.
22. Crespo, J.F., Pascual, C., Burks, A.W., Helm, R.M. & Esteban, M.M. (1995) Frequency of food allergy in pediatric population from Spain. *Pediatr Allergy Immunol* **6**, 39–43.
23. Iikura, Y., Imai, Y., Imai, T. *et al*. (1999) Frequency of immediate-type food allergy in children in Japan. *Int Arch Allergy Immunol* **118**, 251–252.
24. Host, A. & Halken, S. (1990) A prospective study of cow milk allergy in Danish infants during the first 3 years of life. *Allergy* **45**, 587–596.

25. Sampson, H.A. (1999) Food allergy. Part 2. Diagnosis and management. *J Allergy Clin Immunol* **103**, 981–989.
26. Sicherer, S.H. (2002) Clinical update on peanut allergy. *Ann Allergy Asthma Immunol* **88**, 350–361.
27. Pastorello, E.A., Stocchi, L., Pravettoni, V. *et al.* (1989) Role of the elimination diet in adults with food allergy. *J Allergy Clin Immunol* **84**, 475–483.
28. Breiteneder, H. & Ebner, C. (2000) Molecular and biochemical classification of plant derived food allergens. *J Allergy Clin Immunol* **106**, 27–36.
29. Eriksson, N.E. (1982) Food hypersensitivity in patients with pollen allergy. *Allergy* **37**, 437–443.
30. Anderson, L.B., Dreyfuss, E.M., Logan, J., Johnstone, D.E. & Glaser, J. (1970) Melon and banana sensitivity coincident with ragweed pollinosis. *J Allergy* **45**, 310–319.
31. Hernández, J., García Sellés, F.J., Pagán, J.A. & Negro, J.M. (1985) Hipersensibilidad a frutas y verduras y polinosis. *Allergol Immunopathol* **13**, 197–211.
32. Ortolani, C., Ispano, M., Pastorello, E.A., Bigi, A. & Ansaloni, R. (1988) The oral allergy syndrome. *Ann Allergy* **61**, 47–52.
33. Ortolani, C., Pastorello, E.A., Farioli, L. *et al.* (1993) IgE mediated allergy from vegetable allergens. *Ann Allergy* **71**, 470–476.
34. Wüthrich, B. & Dietschi, R. Das (1985) "Sellerie-Karotten-Beifuss-Gewürz-Syndrom". Hauttest und RAST Ergebnisse. *Schweiz Med Wschr* **115**, 358–364.
35. Pauli, G., Bessot, J.C., Braun, P.A., Dietemann-Molard, A., Kopferschmitt-Kubler, M.C. & Thierry, R. (1988) Celery allergy: Clinical and biological study of 20 cases. *Ann Allergy* **60**, 243–246.
36. Blanco, C., Carrillo, T., Castillo, R., Quiralte, J. & Cuevas, M. (1994) Latex allergy: clinical features and cross-reactivity with fruits. *Ann Allergy* **73**, 309–314.
37. Pastorello, E.A., Incorvaia, C., Pravettoni, V. & Ortolani C. (1997) Crossreactions in food allergy. *Clin Rev Allergy Immunol* **15**, 415–427.
38. Epidemiology: Prevalence of allergic diseases. In: *European Allergy White Paper. Allergic diseases as a public health problem in Europe*. Edited by The UCB Institute of Allergy, Braine-l'Alleud, Belgium, 1997, pp. 14–47.
39. Bircher, A.J., van Melle, G., Haller, E., Curty, B. & Frei, P.C. (1994) IgE to food allergens are highly prevalent in patients allergic to pollens, with and without symptoms of food allergy. *Clin Exp Allergy* **24**, 367–374.
40. Bock, S.A. & Atkins, F.M. (1990) Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges. *J Pediatr* **117**, 561–567.
41. Yocum, M.W. & Kahan, D.A. (1994) Assessment of patients who have experienced anaphylaxis: a 3 year survey. *Mayo Clin Proc* **69**, 16–23.
42. Pumphrey, R.S.H. (2000) Lessons for management of anaphylaxis from a study of fatal reactions. *Clin Exp Allergy* **30**, 1144–1150.
43. Sampson, H.A. (1998) Fatal food induced anaphylaxis. *Allergy* **53**, 125–130.
44. Sampson, H.A., Mendelson, L.M. & Rosen, J.P. (1992) Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N Engl J Med* **327**, 380–384.
45. Pastorello, E.A., Rivolta, M.D.F., Bianchi, M.D.V., Mauro, M. & Pravettoni, V. (2001) Incidence of anaphylaxis in the emergency department of a general hospital in Milan. *J Chromatogr B* **756**, 11–17.
46. Foucard, T. & Malmheden Yman, I. (1999) A study on severe food reactions in Sweden. Is soy protein an underestimated cause of food anaphylaxis? *Allergy* **54**, 261–265.
47. Moneret-Vautrin, D.A., Kanny, G. & Parisot, L. (2002) First survey from the "Allergy Vigilance Network": life-threatening food allergies in France. *Allergy Immunol* **34**, 194–198.
48. Macdougall, C.F., Cant, A.J. & Colver, A.F. (2002) How dangerous is food allergy in childhood? The incidence of severe and fatal allergic reactions across the UK and Ireland. *Arch Dis Child* **86**, 236–239.
49. Fernández Rivas, M., van Ree, R. & Cuevas, M. (1997) Rosaceae fruit allergy without related pollinosis. *J Allergy Clin Immunol* **100**, 728–733.
50. Kidd, J., Cohen, S.H., Sosman, A.J. & Fink, J.N. (1982) Food dependent exercise induced anaphylaxis. *J Allergy Clin Immunol* **69**, 103–111.
51. Burks, W., James, J.M., Hiegel, A. *et al.* (1998) Atopic dermatitis and food hypersensitivity reactions. *J Pediatr* **132**, 132–136.
52. de Maat-Bleeker, F. & Bruijnzeel-Koomen, C. (1996) Food allergy in adults with atopic dermatitis. *Monogr Allergy*. Karger, Basel, **32**, 157–163.
53. Sicherer, S.H. & Sampson, H.A. (1999) Food hypersensitivity and atopic dermatitis: Pathophysiology, epidemiology, diagnosis, and management. *J Allergy Clin Immunol* **104**, S114–122.
54. Amlot, P.L., Kemeny, D.M., Zachary, C., Parkes, P. & Lessof, M.H. (1987) Oral allergy syndrome (OAS): symptoms of IgE mediated hypersensitivity to foods. *Clin Allergy* **17**, 33–42.

55. Pastorello, E.A., Ortolani, C., Farioli, L. *et al*. (1994) Allergenic cross-reactivity among peach, apricot, plum, and cherry in patients with oral allergy syndrome: An *in vivo* and *in vitro* study. *J Allergy Clin Immunol* **94**, 699–707.
56. Sánchez-Monge, R., Lombardero, M., García-Sellés, F.J., Barber, D. & Salcedo, G. (1999) Lipid-transfer proteins are relevant allergens in fruit allergy. *J Allergy Clin Immunol* **103**, 514–519.
57. Pastorello, E.A., Farioli, L., Pravettoni, V. *et al*. (1999) The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *J Allergy Clin Immunol* **103**, 520–526.
58. Asero, R., Mistrello, G., Roncarolo, D. *et al*. (2000) Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* **122**, 20–32.
59. De Martino, M., Novembre, E., Cozza, G., de Marco, A., Bonazza, P. & Vierucci, A. (1988) Sensitivity to tomato and peanut allergens in children monosensitized to grass pollen. *Allergy* **43**, 206–213.
60. Valenta, R., Duchène, M., Ebner, C. *et al*. (1992) Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* **175**, 377–385.
61. Van Ree, R., Voitenko, V., van Leeuwen, W.A. & Aalberse, R.C. (1992) Profilin is a crossreactive allergen in pollen and vegetable foods. *Int Arch Allergy Immunol* **98**, 97–104.
62. Vallier, P., Dechamp, C., Valenta, R., Vial, O. & Deviller, P. (1992) Purification and characterization of an allergen from celery immunochemichally related to an allergen present in several other plant species. Identification as profilin. *Clin Exp Allergy* **22**, 774–782.
63. Van Ree, R., Fernández-Rivas, M., Cuevas, M., van Wijngaarden, M. & Aalberse, R.C. (1995) Pollen related allergy to peach and apple: An important role for profilin. *J Allergy Clin Immunol* **95**, 726–734.
64. Ebner, C., Hirschwehr, R., Bauer, L. *et al*. (1995) Identification of allergens in fruit and vegetables: IgE cross-reactivities with the important birch pollen allergens *Bet v* 1 and *Bet v* 2 (birch profilin). *J Allergy Clin Immunol* **95**, 962–969.
65. Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Laimer da Camara Machado, M. *et al*. (1995) Cloning and sequencing of *Mal d* 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to *Bet v* 1, the major birch pollen allergen. *Biochem Biophys Res Commun* **214**, 538–551.
66. Fritsch, R., Ebner, C. & Kraft, D. (1997) Allergenic crossreactivities: Pollens and vegetable foods. *Clin Rev Allergy Immunol* **15**, 397–404.
67. James, J.M., Bernhisel-Broadbent, J., Sampson, H.A. (1994) Respiratory reactions provoked by double-blind food challenges in children. *Am J Respir Crit Care Med* **149**, 59–64.
68. James, J.M., Eigenmann, P.A., Eggleston, P.A. & Sampson, H.A. (1996) Airway reactivity changes in food-allergic, asthmatic children undergoing double-blind placebo-controlled food challenges. *Am J Respir Crit Care Med* **153**, 597–603.
69. Aas, K. (1987) Fish allergy and the codfish allergen model. In: *Food Allergy and Intolerance*, Brostoff, J. & Challacombe, S. (eds). London, Billière Tindal, pp. 356–366.
70. Martín, J.A., Compaired, J.A., de la Hoz, B. *et al*. (1992) Bronchial asthma induced by chick pea and lentil. *Allergy* **47**, 185–187.
71. Crespo, J.F., Pascual, C., Domínguez, C., Ojeda, I., Muñoz, F.M. & Martín Esteban, M. (1995) Allergic reactions associated with airborne fish particles in IgE-mediated fish hypersensitive patients. *Allergy* **50**, 257–261.
72. Lehrer, S.B., Ibáñez, M.D., McCants, M.D., Daul, C.B. & Morgan, J.E. (1990) Characterization of water soluble shrimp allergens released during boiling. *J Allergy Clin Immunol* **85**, 1005–1013.
73. O'Neil Carol, Lehrer, S.B. (1997) Occupational reactions to food allergens. In: *Food Allergy: Adverse Reactions to Foods and Food Additives*, Metcalfe, D.D., Sampson, H.A. & Simon, R.A. (eds). Cambridge, Mass: Blackwell Science, pp. 311–335.
74. Cartier, A., Malo, J.L., Forest, F., Poitras, B. & Rivard, C.H. (1984) Occupational asthma in snow crab processing workers. *J Allergy Clin Immunol* **74**, 261–269.
75. Bernstein, D.I., Smith, A.B. & Moller, D. (1987) Clinical and immunological studies among egg-processing workers with occupational asthma. *J Allergy Clin Immunol* **80**, 791–797.
76. Block, G., Tse, K.S., Kijek, K., Chan, H. & Chan-Yeung, M. (1983) Baker's asthma. Clinical and immunological studies. *Clin Allergy* **13**, 359–370.
77. Osterman, K., Zetterstrom, O. & Johansson, S.G.O. (1982) Coffee worker's allergy. *Allergy* **37**, 313–322.
78. Antó, J.M., Sunyer, J., Rodríguez Roisín, R., Suárez Cervera, M. & Vázquez, L. (1989) Community outbreaks of asthma associated with inhalation of soybean dust. *N Engl J Med* **320**, 1097–1102.
79. De la Hoz, B., Fernández Rivas, M., Quirce, S. *et al*. (1991) Swiss-chard hypersensitivity. Clinical and immunological studies. *Ann Allergy* **67**, 487–492.
80. Igea, J.M., Fernández Rivas, M., Quirce, S., De la Hoz, B. & Díez, M.L. (1994) Green bean hypersensitivity: An occupational allergy in a homemaker. *J Allergy Clin Immunol* **94**, 33–35.

81. Maibach, H. (1976) Immediate hypersensitivity in hand dermatitis. Role of food-contact dermatitis. *Arch Dermatol* **112**, 1289–1291.
82. Von Krogh, G. & Maibach, H.I. (1981) The contact urticaria syndrome. An updated review. *J Am Acad Dermatol* **5**, 328–342.
83. Hjorth, N. & Roed-Petersen, J. (1976) Occupational protein contact dermatitis in food handlers. *Contact Dermatitis* **2**, 28–42.
84. Bock, S., Buckley, J., Holst, A. & May, C. (1978) Proper use of skin tests with food extracts in diagnosis of food hypersensitivity. *Clin Allergy* **8**, 559–564.
85. Malling, H.J. (1993) Methods of skin testing, In: Dreborg, S. & Frew, A. (eds). Position paper: allergen standardization and skin tests. *Allergy* **48** (Suppl 1).
86. Sampson, H.A. & Albergo, R. (1984) Comparison of results of skin tests, RAST, and double-blind, placebo-controlled food challenges in children with atopic dermatitis. *J Allergy Clin Immunol* **74**, 26–33.
87. Sampson, H.A. (1988) Comparative study of commercial food antigen extracts for the diagnosis of food hypersensitivity. *J Allergy Clin Immunol* **82**, 718–726.
88. Sampson, H.A. & Ho, D.G. (1997) Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. *J Allergy Clin Immunol* **100**, 444–451.
89. Sampson, H.A. (2001) Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J Allergy Clin Immunol* **107**, 891–896.
90. Bock, S.A. (1997) *In vivo* diagnosis: Skin testing and oral challenge procedures. In: *Food Allergy: Adverse Reactions to Foods and Food Additives*, Metcalfe, D.D., Sampson, H.A. & Simon, R.A. (eds). Cambridge, Mass, Blackwell Science, pp. 151–166.
91. Ortolani, C., Ispano, M., Pastorello, E.A., Ansaloni, R. & Magri, G.C. (1989) Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. *J Allergy Clin Immunol* **83**, 683–690.
92. Cuesta-Herranz, J., Lázaro, M., Martínez, A. *et al.* (1998) A method for quantification of food biologic activity: Results with peach allergen extracts. *J Allergy Clin Immunol* **102**, 275–280.
93. Rodríguez, J., Crespo, J.F., López-Rubio, A. *et al.* (2000) Clinical cross-reactivity among foods of the Rosaceae family. *J Allergy Clin Immunol* **106**, 183–189.
94. Dreborg, S. & Foucard, T. (1983) Allergy to apple, carrot and potato in children with birch pollen allergy. *Allergy* **38**, 167–172.
95. Aalberse, R.C., Koshte, V. & Clemens, J.G.J. (1981) Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. *J Allergy Clin Immunol* **68**, 356–364.
96. Mari, A., Iacovacci, P., Afferni, C. *et al.* (1999) Specific IgE to cross-reactive carbohydrate determinants strongly affect the *in vitro* diagnosis of allergic diseases. *J Allergy Clin Immunol* **103**, 1005–1011.
97. García-Sellés, F.J., Díaz-Perales, A., Sánchez Monge, R. *et al.* (2002) Pattern of reactivity to lipid transfer proteins of plant foods and *Artemisia* pollen: An *in vivo* study. *Int Arch Allergy Immunol* **128**, 115–122.
98. Son, D.Y., Scheurer, S., Haustein, D. & Vieths, S. (1999) Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *Eur J Nutr* **38**, 201–215.
99. Karamloo, F., Scheurer, S., Wangorsch, A., May, S., Haustein, D. & Vieths, S. (2001) Pyr c 1, the major allergen from pear (*Pyrus communis*), is a new member of the *Bet v* 1 allergen family. *J Chromatogr B* **756**, 281–293.
100. Scheurer, S., Pastorello, E.A., Wangorsch, A., Kästner, M., Haustein, D. & Vieths, S. (2001) Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the *in vitro* diagnosis of cherry allergy. *J Allergy Clin Immunol* **107**, 724–731.
101. Ballmer-Weber, B., Scheurer, S., Fritsche, P. *et al.* (2002) Component-resolved diagnosis with recombinant allergens in patients with cherry allergy. *J Allergy Clin Immunol* **110**, 167–173.
102. Lorenz, A.R., Scheurer, S., Haustein, D. & Vieths, S. (2001) Recombinant food allergens (Review). *J Chromatogr B* **756**, 255–279.
103. Bock, S.A., Sampson, H.A., Atkins, F.M. *et al.* (1988) Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. *J Allergy Clin Immunol* **82**, 986–997.
104. Metcalfe, D.D. & Sampson, H.A. (1990) Workshop on experimental methodology for clinical studies of adverse reactions to foods and food additives. *J Allergy Clin Immunol* **86**, 421–442.
105. Huijbers, G.B., Colen, A.A.M., Jansen, J.J.N., Kardinaal, A.F.M., Vlieg-Boerstra, B.J. & Martens, B.P.M. (1994) Masking foods for food challenge: Practical aspects of masking foods for a double-blind, placebo-controlled food challenge. *J Am Diet Assoc* **94**, 645–649.
106. Noé, D., Bartemucci, L., Mariani, N. & Cantari, D. (1998) Practical aspects of preparation of foods for double-blind, placebo-controlled food challenge. *Allergy* **53** (Suppl 46), 75–77.

107. Smith, L.J. & Muñoz-Furlong, A. (1997) The management of food allergy. In: *Food Allergy: Adverse Reactions to Foods and Food Additives*, Metcalfe, D.D., Sampson, H.A. & Simon, R.A. (eds). Cambridge, Mass, Blackwell Science, pp. 431–444.
108. Bernhisel-Broadbent, J.A. & Sampson, H.A. (1989) Cross-allergenicity in the legume botanical family in children with food hypersensitivity. *J Allergy Clin Immunol* **83**, 435–440.
109. Bernhisel-Broadbent, J.A. (1995) Allergenic cross-reactivity of foods and characterization of food allergens and extracts. *Ann Allergy Asthma Immunol* **75**, 295–303.
110. Ewan, P.W. & Clark, A.T. (2001) Long-term prospective observational study of patients with peanut and nut allergy after participation in a management plan. *Lancet* **357**, 111–115.
111. Moneret-Vautrin, D.A., Kanny, G., Morisset, M. *et al*. (2001) Food anaphylaxis in schools: evaluation of the management plan and the efficiency of the emergency kit. *Allergy* **56**, 1023–1025.
112. Graham, M. & Blaiss, M.S. (2000) Quality of life outcomes measures of asthma and allergic rhinitis. *Pediatr Ann* **29**, 438–443.
113. Meltzer, E.O. (2001) Quality of life in adults and children with allergic rhinitis. *J Allergy Clin Immunol* **108**, 545–553.
114. Baiardini, I., Pasquali, M., Giardini, A., Majani, G. & Canonica, G.W. (2001) Quality of life in respiratory allergy. *Allergy Asthma Proc* **22**, 177–181.
115. Sheikh, A. & Alves, B. (2000) Hospital admissions for acute anaphylaxis: time trend study. *Br Med J* **320**, 1441.
116. Wilson, R. (2000) Upward trend in acute anaphylaxis continued in 1998–9. *Br Med J* **321**, 1021–1022.
117. Gowland, M.H. (2002) Food allergen avoidance: risk assessment for life. *Proc Nutr Soc* **61**, 39–43.
118. Mandell, D., Curtis, R., Gold, M. & Hardie, S. (2002) Families coping with a diagnosis of anaphylaxis in a child. *ACI International* **14**, 96–101.
119. Kemp, A.S. (1999) Atopic eczema: its social and financial costs. *J Pediatr Child Health* **35**, 229–231.
120. Primeau, M.N., Kagan, R., Joseph, L. *et al*. (2000) The psychological burden of peanut allergy as perceived by adults with peanut-allergic children. *Clin Exp Allergy* **30**, 1135–1143.
121. Sicherer, S.H., Noone, S.A. & Muñoz-Furlong, A. (2001) The impact of childhood food allergy on quality of life. *Ann Allergy Asthma Immunol* **87**, 461–464.
122. Calsbeek, H., Rijken, M., Bekkers, M.J., Kerssens, J.J., Dekker, J., van Berge Henegouwen, G.P. (2002) Social position of adolescents with chronic digestive disorders. *Eur J Gastroenterol Hepatol* **14**, 543–549.
123. Fineman, S.M. (2002) The burden of allergic rhinitis: beyond dollars and cents. *Ann Allergy Asthma Immunol* **88**, 2–7.
124. Fireman, P. (1997) Treatment of allergic rhinitis: effect on occupation productivity and work force costs. *Allergy Asthma Proc* **18**, 63–67.
125. Ward, M.M., Javitz, H.S., McFate Smith, W. & Whan, M.A. (2002) Lost income and work limitations in persons with chronic respiratory disorders. *J Clin Epidemiol* **55**, 260–268.
126. European Allergy White Paper. The UCB Institute of Allergy, 1998.
127. Taylor, S.L. & Hefle, S.L. (2001) Ingredient and labelling issues associated with allergenic foods. *Allergy* **56** (Suppl 67), 64–69.
128. Steinman, H.A. (1996) "Hidden" allergens in foods. *J Allergy Clin Immunol* **98**, 241–250.
129. Joshi, P., Mofidi, S. & Sicherer, S.H. (2002) Interpretation of commercial food ingredient labels by parents of food-allergic children. *J Allergy Clin Immunol* **109**, 1019–1021.
130. Sadler, M. & Gatenby, S. (2001) Industry labelling guidelines for allergens and food safety advice. *Nutr Food Sci* **31**, 79–83.
131. McCabe, M., Lyons, R.A., Hodgson, P., Griffiths, G. & Jones, R. (2001) Management to peanut allergy (letter). *Lancet* **357**, 1531–1532.
132. Food Standards Agency (2002) May contain labelling – The consumers perspective. Available from the Food Standards Agency, London.
133. Schäppi, G.F., Konrad, V., Imhof, D., Etter, R. & Wüthrich, B. (2001) Hidden peanut allergens detected in various foods: findings and legal measures. *Allergy* **56**, 1216–1220.
134. Eigenmann, P.A. & Zamora, S.A. (2002) An internet-based survey on the circumstances of food-induced reactions following the diagnosis of IgE-mediated food allergy. *Allergy* **57**, 449–453.
135. Nowak-Wegrzyn, A., Conover-Walker, M.K. & Wood, R.A. (2001) Food-allergic reactions in schools and preschools. *Arch Pediatr Adolesc Med* **155**, 790–795.
136. Rhim, G.S. & McMorris, M.S. (2001) School readiness for children with food allergies. *Ann Allergy Asthma Immunol* **86**, 172–176.
137. Hourihane, J.O'B. (2001) The threshold concept in food safety and its applicability to food allergy. *Allergy* **56** (Suppl 67), 86–90.
138. Nieborg-Eshuis, N.H. (1999) Practical aspects of food allergy and food intolerance. A consumer's perspective. *Bibl Nutr Dieta* **48**, 138–148.

139. Woods, R.K., Stoney, R.M., Raven, J., Walters, E.H., Abramson, M. & Thien, F.C.K. (2002) Reported adverse food reactions overestimate true food allergy in the community. *Eur J Clin Nutr* **56**, 31–36.
140. Brugman, E., Meulmeester, J.F., Spee-van der Wekke, A., Beuker, R.J., Radder, J.J. & Verloove-Vanhorick, S.P. (1998) Prevalence of self-reported food hypersensitivity among school children in the Netherlands. *Eur J Clin Nutr* **52**, 577–581.
141. Knibb, R.C., Booth, D.A., Platts, R., Armstrong, A., Booth, I.W. & MacDonald, A. (2002) Consequences of perceived food intolerance for welfare, lifestyle, and food choice practices, in a community sample. *Psychol Health Med* **5**, 419–430.
142. Eggesbo, M., Botten, G. & Stigum, H. (2001) Restricted diets in children with reactions to milk and egg perceived by their parents. *J Pediatr* **139**, 583–587.

# 2 The Classification, Functions and Evolutionary Relationships of Plant Proteins in Relation to Food Allergies

PETER R. SHEWRY, JOHN A. JENKINS, FREDERIC BEAUDOIN
and E.N. CLARE MILLS

## 2.1 Introduction

Plants synthesise a vast array of proteins which perform a wide range of functions. For example, the recently completed sequences of the genomes of *Arabidopsis* (a *model* plant species which is related to cultivated brassicas including oilseed rape) and rice show the presence of about 25 500 and 50 000 genes, respectively, each encoding a protein [1–3]. However, this number does not include small proteins of less than 100 amino acid residues as these are not predicted by programs used to identify genes in DNA sequences. Furthermore, both *Arabidopsis* and rice were selected as model species because of their small genome size, about $115 \times 10^6$ base pairs (bp) of DNA in *Arabidopsis* and $430 \times 10^6$ bp in rice, compared with the massive genomes of other cultivated species such as oilseed rape ($820 \times 10^6$ bp), maize ($2.5 \times 10^9$ bp) and wheat ($16 \times 10^{12}$ bp). Although much of the additional DNA in the latter species is not transcribed, many cultivated species are either polyploid (e.g. wheat) or derived from ancestral polyploids (e.g. maize), and hence can be expected to have more expressed genes (and hence proteins) than *Arabidopsis* or rice.

Although the maximum number of proteins present in plants can be estimated based on the total number of genes, many proteins are only expressed in trace amounts or in specific tissues or at defined stages of development. For example, about 17% of the genes with *identified functions* (i.e. based on homology of the encoded proteins) in *Arabidopsis* are involved in gene transcription and 10.4% in signalling processes. These would be expected to be present only transiently and/or at low levels.

Consequently, the total number of proteins which can be identified by high-resolution electrophoretic analysis of a given plant tissue is usually numbered in hundreds rather than in thousands. For example, Clarke *et al.* [4] estimated that between 4500 and 8000 genes are active in the developing wheat grain but only 1697 proteins were identified by two-dimensional electrophoresis. Furthermore, in a similar study, Skylas *et al.* [5] identified only 321 proteins which were present at levels sufficient to allow their excision and analysis by *N*-terminal sequencing. It is this relatively small number of quantitatively major proteins which determine the end-use quality of a plant tissue and which have the potential to be allergenic.

## 2.2 Plant proteins in nutrition and processing

Both the amount and composition of plant proteins have impacts on human nutrition. Adult men and women have been estimated to require 0.8 g protein/kg body weight

per day, with higher requirements for infants and children. Plant proteins may provide a significant proportion of this intake, particularly in developing countries, but also increasingly in diet-conscious Western European and other developed countries. Because fruit and vegetables contain little protein on a fresh-weight basis, much of the plant protein consumed by humans is from protein-rich storage organs, chiefly seeds but to a lesser extent tubers. Seeds contain specialised types of storage proteins which may account for 50% or more of the total proteins in the organ. However, these proteins frequently have an unbalanced composition of amino acids, with the proportions of some essential amino acids falling below the minima recommended by nutritionists. In general, cereal seeds are deficient in the essential amino acid lysine and, to a lesser extent, threonine and tryptophan, while pulses and other legume seeds are deficient in the sulphur-containing amino acids cysteine and methionine. Combining these two types of seed results in a balanced diet and this strategy is often used when formulating diets for monogastric livestock.

Although nutritional quality for humans is a major consideration in countries where the diet is largely plant-based, the most important impact of plant proteins on human food consumption in developed countries is their role in food processing.

A high proportion of the food consumed in developed countries is in processed form and plant proteins are crucial in conferring the functional properties which make this possible. In particular, soybean proteins can be textured to form meat analogues or used to confer a range of important properties including gelation (e.g. in tofu), foaming, emulsification, water absorption, fat absorption, viscosity, elasticity and flavour binding [6]. Consequently, soybean protein is remarkably pervasive in food systems. Although used in a narrower range of products than soybean proteins, wheat grain proteins are even more important in food processing as they confer the visco-elastic properties that enable dough to be made into bread, other baked goods, pasta and noodles. Wheat flour and isolated gluten are also used in other food products such as sauces, batters and processed meat. Consequently, wheat products are an integral part of the diet in many countries, with a high proportion of the total world production of wheat being consumed by humans in processed form.

Consequently, humans encounter a wide range of plant proteins in their diets – from native proteins in fresh fruits and vegetables to highly modified seed proteins in processed foods.

## 2.3 The classification of plant proteins

### 2.3.1 *Osborne fractions based on solubility*

Plant proteins were among the first to be studied, with the isolation of wheat gluten being described in 1745 [7]. Subsequent work led to the concept of classifying plant proteins according to their solubility, and this was formalised by T.B. Osborne who published studies of proteins from 32 plant species between 1886 and 1928 [8]. Osborne classified proteins into four groups based on their solubility in water (albumins), dilute salt solutions (globulins), alcohol/water mixtures (typically 60–70%

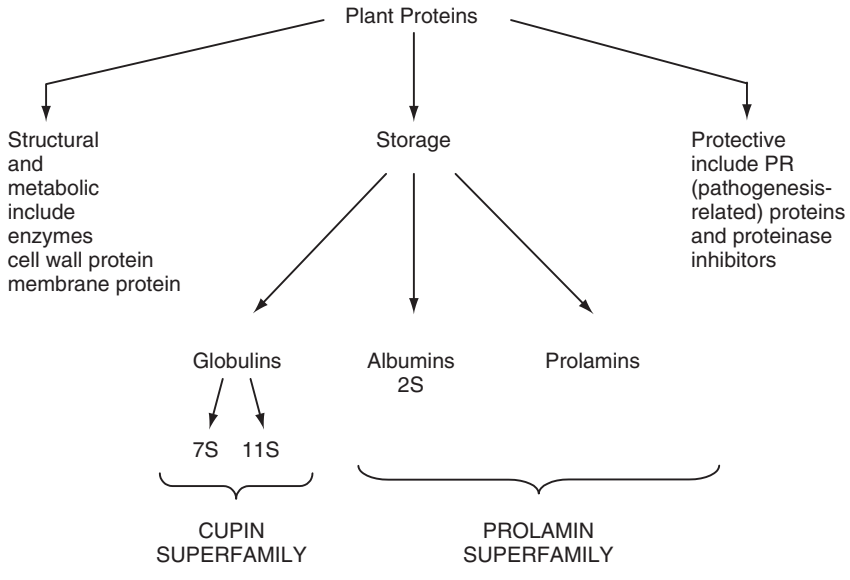


**Fig. 2.1** Summary of the classification of plant proteins.

(v/v) ethanol) and dilute alkali (glutelins), and it has subsequently become usual to extract these fractions sequentially, a procedure called Osborne fractionation. Few modern plant scientists are aware of Osborne's work and of his classification system, although the terms albumin and globulin remain widely used for proteins from seeds and other tissues. However, the Osborne classification is still used by those working on seed proteins, particularly by cereal chemists. This is because the Osborne groups extracted from seeds may be highly enriched in specific storage proteins and hence represent biologically and functionally valid fractions for further analysis.

In seeds of legumes and many other dicotyledonous plants, the globulin fractions are highly enriched in storage proteins and the term *legume globulin* is often used as synonym for *legume seed storage protein*. In wheat and most other cereals (but not in oats and rice), the prolamins comprise the major storage protein fraction, but related storage proteins are also present in the glutelin fractions. These *glutelin* storage proteins are now known to exist in the grain as polymers stabilised by inter-chain disulphide bonds, the individual reduced subunits being readily soluble in alcohol–water mixtures with clear relationships to prolamins. Consequently, it is now usual to extract total prolamin fractions from cereal grain using alcohol–water mixtures (usually 50% (v/v) propan-1-ol) containing a reducing agent (usually 2-mercaptoethanol or dithiothreitol).

However, despite the continued use of classification based on solubility by seed scientists, most plant biologists consider that it is now more valid to use alternative classifications, based either on function or structural and evolutionary relationships (see Fig. 2.1).

### 2.3.2 *Functional classification*

Plant proteins can be divided into three broad groups based on their functions.

2.3.2.1 *Structural and metabolic proteins.* These include a vast array of proteins which contribute to the structure and functioning of the cell, including structural components of cell walls and organellar membranes, enzymes, and proteins involved in energy generation, trafficking and transport, growth and division, signal transduction, gene transcription and protein synthesis. Although some of these proteins may be described as *housekeeping*, in that they are present in all cells, others are present only transiently or in specific cell types or stages of development. Similarly, although many are present in small amounts, others are major components. In particular, enzymes and other proteins of the photosynthetic machinery are highly abundant in green tissues, with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme responsible for carbon dioxide fixation, accounting for 30–40% of the total leaf protein in most species. There is no doubt that humans consume vast quantities of Rubisco over a lifetime, in fresh and cooked vegetables. Nevertheless, there is no evidence that either Rubisco or other structural and metabolic proteins are responsible for widespread allergenic reactions.

2.3.2.2 *Protective proteins.* Plants synthesise a wide range of proteins which are thought to contribute to protection against pathogenic micro-organisms (bacteria, fungi) and/or invertebrate pests (insects, nematodes). In most cases, a putative biological role has been ascribed based on their activity *in vitro*, for example as inhibitors of enzymes or as lectins. However, in some cases, this activity has been confirmed by more direct analyses such as incorporation into artificial diets for insect feeding tests, incorporation into media used for culturing micro-organisms or by expression in transgenic plants.

Plant storage organs (seeds and tubers) contain rich reserves of starch, protein and oil that make them attractive to pests and pathogens. It is not surprising, therefore, that they are also rich in protective proteins. Furthermore, the exploitation of storage organs by humankind results in consumption of the protective proteins as well as the storage reserves.

The protective proteins in seeds and tubers are synthesised irrespective of whether the plant is challenged by pests or pathogens. In contrast, protective proteins are often synthesised in vegetative tissues as a response to infection or damage (either accidental or due to feeding), forming part of a *pathogenesis-related* (PR) response [9]. Consequently, they may be present in vegetables which have been damaged in the field or during harvesting.

Limited space precludes a full discussion of protective proteins and the reader is referred to a recent review article [10]. However, they include components with four major types of biological activity.

1. Enzyme inhibitors: Inhibitors of proteinases form the largest and the most diverse type of protective proteins, with at least 12 distinct classes being recognised based on their amino acid sequences and target enzymes [11]. Of these, ten are inhibitors of serine proteinases, the most widespread type of proteinases in animals and microbes. Other protective proteins inhibit α-amylases of insects, mammals and microbes, and polygalacturonase, an enzyme secreted by plant pathogenic fungi.

2. A second major type of protective protein is hydrolases which digest components of microbial cell walls and insect cuticles. These include lysozyme which digests bacterial cell walls, and chitinases and β-glucanases which may be active against fungal hyphae and insect cuticles.

3. A number of structurally unrelated types of defensive proteins appear to destabilise membranes, including hyphal membranes of fungi, leading to leakiness. They may also act synergistically and include thionins, 2S albumin storage proteins, thaumatin-related proteins, lipid transfer proteins (LTPs) and defensins (see chapters in [12]).

4. A number of types of defensive proteins are able to bind to chitin, an α-1,4-linked polymer of *N*-acetyglucosamine, present in cuticles and gut linings of insect and fungal hyphae. These include lectins, hevein, endochitinases (see above) and some defensins. It is notable that several well-characterised classes of allergens appear to have protective roles, as discussed in other chapters in this volume.

2.3.2.3 *Storage proteins.* Storage proteins are most widely characterised from seeds where they are deposited during development to provide a store of amino acids and carbon skeletons for germination and seedling growth. However, storage proteins may also accumulate in vegetative tissues and tubers, in order to support sprouting or to assist survival during periods of adverse conditions (e.g. drought, low temperature).

Vegetative and tuber storage proteins vary widely in their structures, properties and evolutionary relationships, with little apparent relationship between the types of proteins stored in different species or organs. Thus, the storage protein of potato tubers exhibits enzymic activity as a lipid acyl hydrolase, while the analogous protein in yam tubers is carbonic anhydrase and in sweet potato a Kunitz-type proteinase inhibitor. Similarly, the vegetative storage proteins of soybean and *Arabidopsis* appear to be acid phosphatases, although the specific activity of the former is very low, while those in the bark and wood of poplar trees are related to wound-inducible proteins from the same species [13–16]. It is probable, therefore, that different metabolic or protective proteins have assumed storage roles in different plant species and organs.

In contrast, there are clear homologies between seed storage proteins across Angiosperms, with three major types which were defined initially on their solubility properties. The characteristics of these groups are discussed briefly below and summarised in Table 2.1.

*Albumins*: Water-soluble albumin storage proteins with sedimentation coefficients ($S_{20.w}$) of about 2 are widespread in seeds of dicotyledonous plants including cultivated species such as brassicas (e.g. oilseed rape), legumes (pea, lupin, soybean), sunflower, cotton and castor bean [17, 18]. Although they vary considerably in their amino acid sequences, they are typically synthesised as a proprotein which is proteolytically processed to give small ($M_r$ approximately 4000–5000) and large ($M_r$ approximately 9000–10 000) subunits with four disulphide bonds, two between the subunits and two within the large subunit. They include a number of well-characterised allergens and are discussed in detail in a later section.

*Globulins*: Salt-soluble globulin storage proteins are almost universally present in seeds of both monocotyledonous and dicotyledonous plants. They comprise two groups of proteins with sedimentation coefficients of about 7/8 and 11/12 [19].

**Table 2.1** Summary of the properties of typical albumin, globulin and prolamin storage proteins

| | 2S albumins | 7S globulins | 11S globulins | Prolamins |
|---|---|---|---|---|
| Solubility | Water | 0.5–1.0 M NaCl | 0.5–1.0 M NaCl | 60–70% (v/v) ethanol/50% (v/v) propan-1-ol (+ reducing agent) |
| $M_r$ | 10–20 000 | 150–200 000 | 300–400 000 | Monomers of ≅10 000–70 000, polymers may exceed $10 \times 10^6$ |
| Subunit Structure | Single proprotein processed to give two subunits with two inter-chain and two intra-chain S–S bonds. No glycosylation | Trimeric $M_r$ 50–80 000 subunits may be proteolytically processed and/or glycosylated. No disulphide bonds | Hexameric $M_r$ 60 000 subunits proteolytically cleaved to give large (acidic, α) and small (basic, β) chains with one inter-chain S–S bond. No glycosylation. No intra-chain S–S bonds | Monomers with intra-chain S–S bonds or lacking cysteine. Polymers with inter-chain S–S bonds. Polymer structure not defined. No glycosylation |
| Amino acid sequence and composition | Some methionine-rich forms | Deficient in cysteine and methionine | Deficient in cysteine and methionine | Deficient in lysine and threonine or tryptophan. Some methionine-rich forms. Contain repeated sequences which result in high levels of some amino acids |
| Distribution | Embryos and/or endosperms of dicotyledonous seeds | Embryos and/or cotyledons of dicotyledonous seeds. Embryos and aleurone layers of cereal seeds | Embryos and/or cotyledons of dicotyledonous seeds. Starchy endosperm cells of some cereals | Starchy endosperm cells of cereals |

The 11S globulins are often called legumins as they are characteristic of legume seeds and have been studied in the most detail from these species. However, they are actually the most widely distributed group of seed storage proteins, occurring in most dicotyledonous species (including brassicas and composits), and in the cereals oats and rice. Typical legumins are hexameric proteins of $M_r$ 300 000–400 000, comprising six subunits of $M_r$ about 60 000. These subunits are post-translationally processed to give large (also called acid or α) and small (basic or β) chains of $M_r$ approximately 40 000 and 20 000, respectively, which remain associated by a single disulphide bond. In contrast, the 7S globulins are trimeric proteins of $M_r$ approximately 150–200 000. The subunits are typically of $M_r$ 50 000–80 000, but post-translational proteolysis and glycosylation can give rise to much wider variation in mass (e.g. from about 12 000 to 70 000 in pea).

*Prolamins*: Prolamins form the most clearly defined group of seed storage proteins, and have the most restricted distribution. However, they are also the most diverse in structure. Prolamins were initially defined on their solubility in alcohol/water mixtures (60–70% (v/v) ethanol), and their high contents of proline and amide nitrogen (hence the name prolamin). However, we now know that related proteins may be present in the grain as disulphide-stabilised polymers and are only alcohol-soluble as reduced subunits. Consequently, the definition of prolamins has been modified to include those proteins which are soluble in alcohol/water mixtures in the native or reduced state, and in practice other alcohols (e.g. 50% (v/v) propan-1-ol) may be more effective solvents than ethanol. Similarly, the combined contents of proline and glutamine (the main amide) are known to vary from about 30 to 70 mol%. However, the third distinguishing feature of prolamins remains unmodified. This is their distribution, which is restricted to seeds of cereals and other members of the grass family. True prolamins are indeed restricted to grass seeds, but we now know that they form part of a larger protein superfamily which includes proteins with much wider distributions.

It is not possible to provide a detailed discussion of prolamin structure within the confines of this chapter but two features are particularly noteworthy and the reader is referred to chapters in [12] for detailed accounts. The first is that the sequences of most prolamins can be divided into two or more domains, which differ in their amino acid compositions, adopt different three-dimensional structures and may differ in origin. The second is that most prolamins contain one or more domains based on repeated blocks of amino acids, usually based on one, two or three short peptide motifs. Such repeated sequences are responsible for the high levels of glutamine, proline and some other amino acids (e.g. phenylalanine, histidine, glycine) in specific prolamin groups. In addition, other parts of the protein sequence may be enriched in amino acids such as methionine. Further details of prolamin structure are provided below.

## 2.4 Plant protein superfamilies

The classification of plant proteins based on solubility, sedimentation coefficient and function has provided a useful framework for almost a century. However, the

availability of amino acid sequences of proteins from molecular cloning and direct sequence analysis, and of three-dimensional structures from nuclear magnetic resonance spectroscopy and X-ray crystallography have allowed their evolutionary and structural relationships to be determined, leading to a new classification into families and superfamilies.

Many seed proteins, including a number of important allergens, belong to two such superfamilies, called the cereal prolamin superfamily and the cupins. We will, therefore, consider the structures and properties of these in some detail.

### 2.4.1 *The prolamin superfamily*

The highly restricted distribution of prolamins, their unusual solubility properties and their unusually high contents of proline and glutamine led to the view that they were a unique type of protein present only in cereal seeds. This view persisted into the mid-1980s, when the availability of complete amino acid sequences and the development of statistical methods of sequence analysis allowed wider comparisons of protein relationships to be made. This resulted in two surprising findings.

Firstly, prolamins do not form a single family of proteins but comprise two major groups which have no known relationship to each other. The first group comprises the α-zeins of maize and related prolamins of other tropical cereals (millets, sorghum), while the second is a larger group comprising the prolamins of the tribe Triticeae (wheat, rye, barley), oats and rice, and minor prolamins of maize, millets and sorghum. Secondly, while the α-zein group has no known relationship to any other characterised proteins, the second group is clearly related to a range of other plant proteins which have together been defined as the *prolamin superfamily* [20, 21].

The α-zein group of prolamins does not include any known allergens and the reader is referred to recent review articles for further details [22, 23]. In contrast, the prolamin superfamily includes several groups of well-characterised respiratory and food allergens. They are therefore briefly discussed below with further details being provided in other articles in this volume.

2.4.1.1 *Identification and characterisation of the prolamin superfamily.* Cereal prolamins have been the subject of a vast volume of research because of their importance in nutrition and food processing. However, the existence of related proteins was not proposed until 1985, based on the identification of conserved amino acid residues [20, 24]. Although this identification was initially based on visual comparisons, it is supported by more sophisticated computational comparisons. Furthermore, although the initial studies only identified two groups of seed proteins as related to prolamins (2S albumins and cereal α-amylase/trypsin inhibitors), subsequent comparisons have shown homology with several other groups of seed and non-seed proteins. All of these proteins can be described as *low $M_r$ cysteine-rich proteins*. In addition, most of them contain a characteristic conserved pattern of eight cysteine residues which can be defined by the formula:-

$$\text{Cys-(Xaa)}_n\text{-Cys-(Xaa)}_n\text{-Cys-Cys-(Xaa)}_n\text{-Cys-Xaa-Cys-(Xaa)}_n\text{-Cys-(Xaa)}_n\text{-Cys}$$

Although some variation in this cysteine pattern does occur (in the numbers of cysteine residues and in the disulphide bonds that they form), the pattern is sufficiently well conserved to identify related protein groups which have little or no other sequence identity.

Protein groups so far identified as belonging to the prolamin superfamily are listed in Table 2.2 and the overall sequence relationships of the major types are summarised as a dendrogram in Fig. 2.2.

Several of these groups of prolamin-related proteins include major allergens which are discussed in some detail elsewhere in this volume. The present account will, therefore, focus on comparative structures and properties.

The prolamins are immensely variable in their structures and properties, and the reader is referred to chapters in [12] for detailed accounts. Allergies to prolamins do not occur very frequently and have only been studied in wheat where they are involved in wheat-induced atopic dermatitis [25, 26] and exercise-induced anaphylaxis (EIA) [27]. Two types of gliadin (α-and γ-) have been reported as the allergens in EIA [28]. Similarly, α-gliadin, γ-gliadin and low molecular weight (LMW) subunits of glutenin have been shown to bind to IgE fractions from patients with dietary allergy to wheat, with LMW subunits containing the pentapeptide motif Gln.Gln.Gln.Pro.Pro being the most active [29, 30].

α-gliadin, γ-gliadin and LMW subunits are all defined as sulphur-rich prolamins and have similar sequences comprising a repetitive *N*-terminal domain and a non-repetitive *C*-terminal domain, the latter containing the conserved cysteine skeleton which is characteristic of the prolamin superfamily (labelled 1–8 in Fig. 2.3). However, it is notable that while the full skeleton of eight cysteine residues is present in the γ-gliadins, only six of these are present in the α-gliadins and LMW subunits. The latter also have one or two additional cysteine residues (a and g in Fig. 2.3) which form inter-chain disulphide bonds to incorporate the subunits into high molecular mass glutenin polymers. The IgE-binding pentapeptide (Gln.Gln.Gln.Pro.Pro) in allergenic LMW subunits is located within the repetitive domain of the LMW subunits, rather than the non-repetitive domains which are homologous with other members of the prolamin superfamily.

The 2S albumins, non-specific lipid transfer proteins (nsLTPs) and cereal inhibitors of α-amylase and trypsin include major dietary and/or respiratory allergens which are discussed in detail in Chapters 3–5. All are large and complex groups of related proteins with considerable structural diversity. In contrast, the soybean hydrophobic protein, responsible for respiratory allergy to soybean hulls [31, 32], has no apparent close relatives and is not discussed elsewhere in this volume. It comprises 80 amino acid residues with the characteristic-conserved eight cysteine residue skeleton forming four intra-chain disulphide bonds [33, 34].

A relationship between the soybean hydrophobic protein and the 2S albumins, nsLTPs and cereal inhibitors is apparent by comparison of their cysteine skeletons and is even more striking when their three-dimensional structures are compared (Plate 2.1). All of the proteins have a similar fold comprising bundles of α-helices stabilised by disulphide bonds and are classified in the SCOP protein structure database [35] as *4 helices; folded leaf; right-handed super-helix; disulphide-rich*. However, the pattern of disulphide bonds varies between the different members with only two of the four

**Table 2.2** Characteristics of members of the Prolamin superfamily

| Protein family | $M_r$ | Distribution | Characteristics | Characterised allergens | Key references* |
|---|---|---|---|---|---|
| Prolamins | 10–100 000 | Cereal grain endosperms | See Table 2.1 | Dietary | [12] |
| α-amylase/trypsin inhibitors | 12–16 000 | Cereal grain endosperms | Monomeric, dimeric and tetrameric forms; may be bifunctional; may be protective | Dietary and respiratory | [79] |
| Non-specific lipid transfer proteins | 7–9000 | Seeds, fruit, other tissues; monocots and dicots | *In vitro* lipid-binding/transfer activities; may be protective | Dietary | [80] |
| 2S albumins | 14–16 000 | Dicot seeds, fern spores | See Table 2.1 | Dietary | [18] |
| Puroindolines | 13 000 | Cereal grain endosperms | Bind to starch and lipids: Trp-rich motif; may be protective | None | [80] |
| Hydrophobic protein | 9000 | Soybean seeds | Function unknown | Respiratory | [33, 34] |
| α-globulins | 18–26 000 | Cereal grain endosperms | Storage proteins | None | [81] |
| Hydroxyproline-rich proteins (HyPRP) | ≅15–45 000 | Various tissues | Include cell wall glycoproteins and stress-induced proteins | None | [82] |

* See also relevant chapters in this volume.

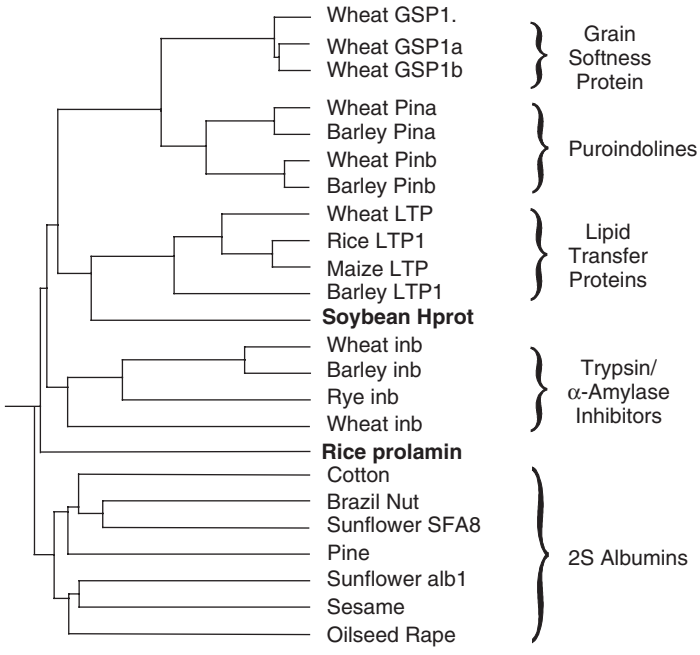


**Fig. 2.2** Dendrogram showing the relationships between the amino acid sequences of small sulphur-rich seed proteins of the prolamin superfamily.

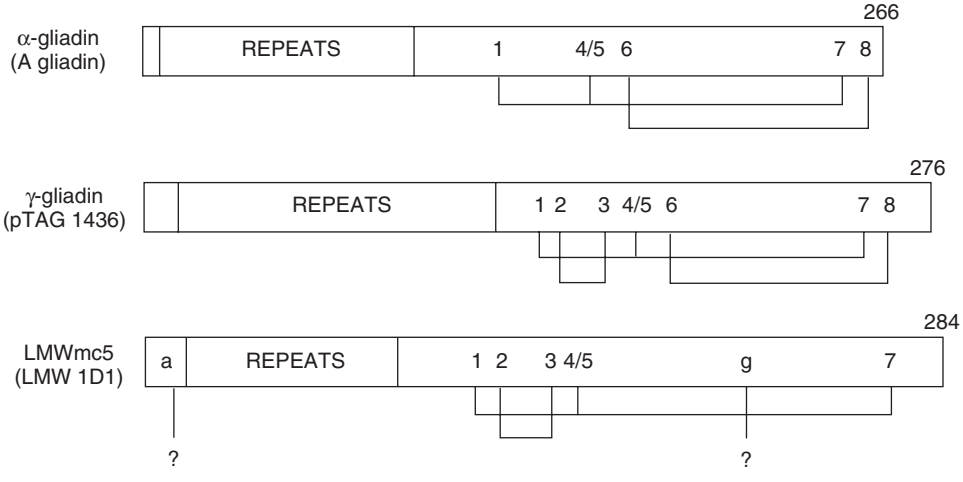


**Fig. 2.3** Schematic comparison of the amino acid sequences of typical types of S-rich prolamin of wheat. Based on sequences reported by Kasarda *et al*. [83], Bartels *et al*. [84] and Colot *et al*. [85]. Taken from [21] with permission. Question marks denote cysteine residues for which no disulphides have been mapped.

bonds being conserved among all of the members. In particular, the Cys-X-Cys motif in helix three of the proteins forms a different pattern of disulphide bonds in the nsLTPs, to those in the other proteins.

### 2.4.2 *The cupin superfamily*

The relationships of the cupin superfamily of proteins have been defined by parallel studies by two groups: Shutov and Baumlein working in Moldova and Germany [36], and Dunwell and colleagues in the UK. Dunwell [37] also coined the term cupin to describe their common structural feature. This is a barrel-like structure which has also been described as a *double-stranded β-helix* [35] or *jellyroll* (Fig. 2.4). (The term cupin being based on the Latin *cupa* meaning a small barrel or cask). The conclusion that this immensely diverse group of proteins have a common origin is based on the demonstration that they share two consensus sequence motifs: $G(x)_5HxH(x)_{3,4}E(x)_6G$ for motif 1 and $G(x)_5PxG(x)_2H(x)_3N$ for motif 2, where X is any amino acid residue but often includes a metal-binding site [38].

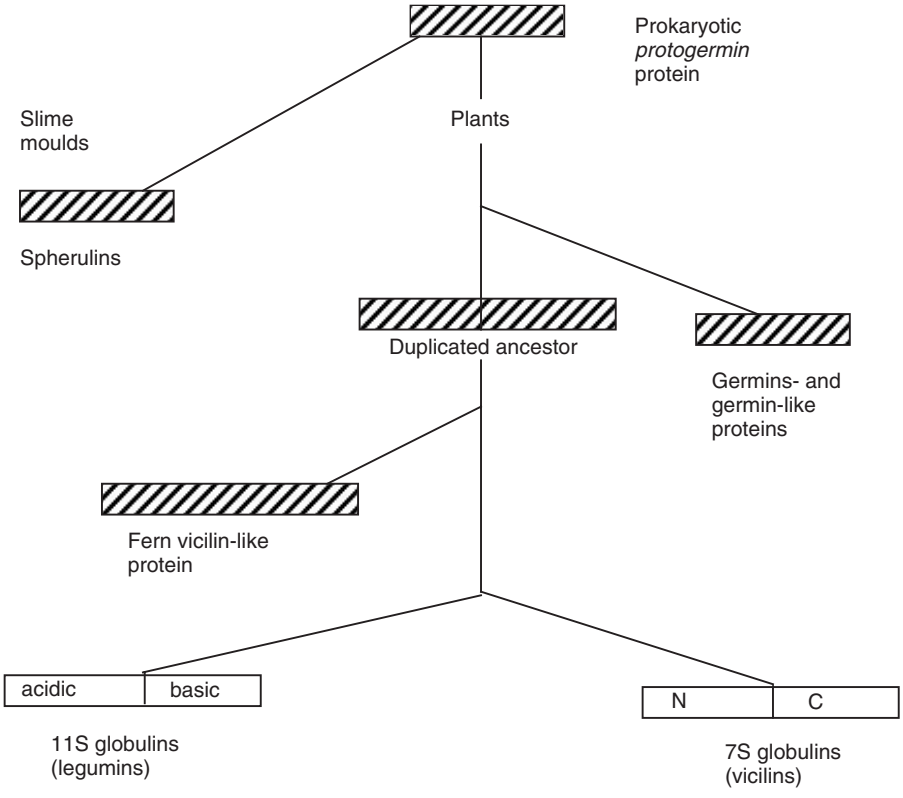


**Fig. 2.4** Hypothetic pathway for the evolution of germins and globulin storage proteins from an ancestral *protogermin* protein, incorporating models of Shutov and Baumlein [36] and Lawrence [44].

Genes encoding cupins have been identified in all organisms whose genomes have been sequenced – Archaea, Eubacteria and Eukaryota [38]. A broad division can be made between proteins with one or two cupin domains, the latter being considered to have arisen from an ancestral duplication event and being termed *bicupins* (Fig. 2.4). The best-characterised cupins from plants are germins and storage globulins, which are single-chain cupins and bicupins, respectively.

The first germin was identified as a protein synthesised in germinating embryos of wheat [39]. It is a soluble pepsin-resistant glycoprotein which exists as an oligomer of $M_r$ about 125 000, with individual subunit $M_r$ of about 25 000. Despite detailed studies, the role of germins remained unknown until they were demonstrated to exhibit activity as the enzyme oxalate oxidase [40]. X-ray cryptallographic analysis shows that each germin monomer contains a single double-stranded β-helix fused to a short α-helical domain (Plate 2.2), with six monomers forming a hexamer which is in effect a *trimer of dimers* [41]. A germin from pepper (*Piper nigrum*) corns (i.e. seeds) has been reported to be an allergen in the cerery–birch–mugwort–spice syndrome [42], but no other allergenic forms have been reported. In contrast, the globulin storage proteins are major allergens in legumes (notably peanut and soybean, see Chapter 9) and other species.

Early studies of the 7S and 11S globulins showed little similarity in structure or properties with the exception of solubility in dilute salt solution. However, it has been observed that the trimeric 7S globulin proteins may reversibly aggregate into hexamers depending on ionic strength. Similarly, although the 11S globulins are typically hexamers, they are initially assembled in the endoplasmic reticulum into trimeric intermediates, and assume the hexameric form only in the vacuole where proteolytic processing of the subunits to give acidic and basic chains also occurs.

Early comparisons of amino acid sequences of 7S and 11S globulin subunits failed to show significant sequence identity. However, more sophisticated comparisons subsequently demonstrated that the *N*- and *C*-terminal regions of the 7S globulin subunits were related to the acidic and basic subunit chains of the 11S globulins, respectively, indicating that they have arisen from a common ancestral gene as shown in Fig. 2.4 [43, 44].

Three-dimensional structures have since been determined for 7S globulins from three species, phaseolin from French bean (*Phaseolus vulgaris*) [45, 46], canavalin from jack bean (*Canavalia ensiformis*) (Plate 2.3) [47, 48], β-conglycinin from soybean [49] and the trimeric 11S globulin precursor proglycinin from soybean [50]. These show a remarkably high degree of similarity as shown in Plate 2.4 in which the backbone structures of phaseolin (7S) and proglycinin (11S precursor) subunits are overlaid.

### 2.4.3 *Cysteine proteinases*

Many plant food allergens either function as or are related to defensive proteins, including components of the PR protein complex. Most of these are described in detail in other chapters in this volume. However, one other major group of allergens which may have a defensive role is the cysteine proteinases.

Cysteine proteinases of the C1 or papain-like family occur widely in plants where they may participate in a range of processes including digestion of storage proteins during seed germination [51–53], senescence of flowers and leaves [54–56], and processing of enzyme precursors [57]. Cysteine proteinases in maize have also been associated with resistance to lepidopteran pests [58, 59], while the high levels of cysteine proteinases in fruits is also consistent with a protective role. The most widely studied cysteine proteinase of plant origin is papain which is present in the latex of papaya (*Carica papaya*). Preparations of papain are widely used in the food industry; e.g. to supplement malt enzymes in brewing, to tenderise meat.

Allergenic cysteine proteinases have been reported from a diverse range of species. The best characterised food allergen is Act c 1 which is the major allergen from kiwi fruit [60–65]. Bromelain from pineapple stems [66], papain from papaya latex and ficin from fig latex [67] are also food allergens. In addition, bromelain and papain are also involved in non-food allergies due to their use in cosmetics and medicine [68, 69], while papain has also been reported to be a pollen allergen [70].

The soybean allergen Gly m Bd 30K was initially described as associated with oil bodies (the P34 or 4-kDa oil-body-associated protein), but is now known to be present in protein storage vacuoles in the seed [71, 72]. The activity of Gly m Bd 30K as a cysteine proteinase has not been demonstrated and an alternative role, binding

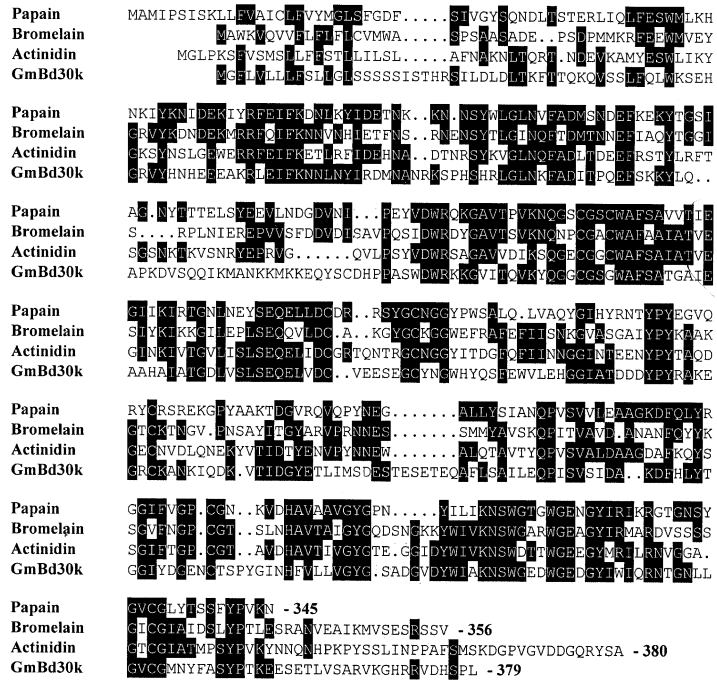


**Fig. 2.5** Comparison of the amino acid sequences of allergenic cysteine proteinases from papaya (papain, Swissprot acc. P00784), pineapple (bromelain, PIR acc. T10514), kiwi fruit (actinidin, Swissprot acc. P00785) and soybean (GmBd40K, Genbank acc. BAA25899).

syringolide elicitors as part of a protective response, has been proposed [73]. Gly m B 30K is a major dietary allergen associated with atopic dermatitis [72].

The mature cysteine proteinases have molecular masses of about 25 000. Their three-dimensional structures (Plate 2.5) contain an α-helical *N*-terminal domain and an α+β *C*-terminal domain which both contribute to the formation of an active site cleft [74, 75]. The mature enzymes are typically stabilised by three disulphide bonds and tend to be stable against denaturation [76].

However, the sequences predicted from cDNAs and genes (Fig. 2.5) are often considerably longer, with an *N*-terminal signal sequence followed by an α-helical prodomain which is necessary for folding and which inactivates the proenzyme by blocking the active site [77, 78]. For example, the papain precursor shown in Plate 2.5 consists of 345 residues of which 1–18 are the signal peptide, 19–133 the prosequence and 134–345 the mature protein.

## 2.5 Conclusions

It is clear that the concept of protein families provides an important framework for the identification and analysis of plant protein allergens, facilitating the prediction of potential allergens and providing a basis for structure–function studies. In particular, comparison of the structures and biological properties of allergenic and non-allergenic members of well-defined protein families should lead to a clearer understanding of the various structural and biological features which together result in allergenicity.

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## References

1. The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796–815.
2. Yu, J., Hu, S., Wang, J. *et al.* (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). *Science* **296**, 79–92.
3. Goff, S.A., Ricke, D., Lan, T.-H. *et al.* (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science* **296**, 92–100.
4. Clarke, B.C., Hobbs, M., Skylas, D. & Appels, R. (2000) Genes active in developing wheat endosperm. *Funct Integr Genomics* **1**, 44–55.
5. Skylas, D.J., Mackintosh, J.A., Cordwell, S.J. *et al.* (2000) Proteome approach to the characterisation of protein composition in the developing and mature wheat-grain endosperm. *J Cereal Sci* **32**, 169–188.
6. Kinsella, J.E. (1979) Functional properties of soy protein. *J American Oil Chemists Soc* **56**, 242–258.
7. Beccari, J. (1745) De Frumento. *De Bononiensi Scientiarum et Artium Instituto atque Academia Commentarii, II*. Part 1, pp. 122–127.
8. Osborne, T.B. (1924) *The Vegetable Proteins*, 2nd edn. Longmans, Green and Co., London, p. 154.
9. van Loon, L.C. & van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities and comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol* **55**, 85–97.

10. Shewry, P.R. & Lucas, J.A. (1997) Plant proteins that confer resistance to pests and pathogens. In: *Advances in Botanical Research*, Vol. 26, Callow, J. (ed.). Academic Press, pp. 135–192.
11. Shewry, P.R. (1999) Enzyme inhibitors of seeds: types and properties. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 587–615.
12. Shewry, P.R. & Casey, R. (eds) (1999) *Seed Proteins*. Kluwer Academic Publishers, Dordrecht, The Netherlands, p. 883.
13. Stanwick, P.E. (1990) Novel regulation of vegetative storage protein genes. *Plant Cell* **2**, 1–6.
14. Berger, S., Bell, E., Sadka, A. & Mullet, J.E. (1995) *Arabidopsis thaliana Atvsp* is homologous to soybean *VspA* and *VspB*, genes encoding vegetative storage protein acid phosphatases, and is regulated similarly by methyl jasmonate, wounding, sugars, light and phosphate. *Plant Mol Biol* **27**, 933–942.
15. Coleman, G.D., Chen, T.H.H. & Fuchigami, L.H. (1992) Complementary DNA cloning of poplar bark storage protein and control of its expression by photoperiod. *Plant Physiol* **98**, 687–693.
16. Clausen, S. & Appel, K. (1991) Seasonal changes in the concentration of the major storage protein and its mRNA in xylem ray cells of poplar trees. *Plant Mol Biol* **17**, 669–678.
17. Youle, R.J. & Huang, A.H. (1981) Occurrence of low molecular weight and high cysteine containing albumin storage proteins in oilseeds of diverse species. *Am J Bot* **68**, 44–48.
18. Shewry, P.R. & Pandya, M.J. (1999) The 2S albumin storage proteins. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 563–587.
19. Casey, R. (1999) Distribution and some properties of seed globulins. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 159–169.
20. Kreis, M., Forde, B.G., Rahman, S., Miflin, B.J. & Shewry, P.R. (1985) Molecular evolution of the seed storage proteins of barley, rye and wheat. *J Mol Biol* **183**, 499–502.
21. Shewry, P.R., Tatham, A.S. & Halford, N.G. (1999) The Prolamins of the Triticeae. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 35–78.
22. Coleman, C.E. & Larkins, B.A. (1999) The Prolamins of Maize. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 109–139.
23. Leite, A., Neto, G.C., Vettore, A.L., Yunes, J.A. & Arruda, P. (1999) The Prolamins of Sorghum, *Coix* and Millets. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 141–157.
24. Kreis, M., Shewry, P.R., Forde, B.G., Forde, J. & Miflin, B.J. (1985) Structure and evolution of seed storage proteins and their genes, with particular reference to those of wheat, barley and rye. In: *Oxford Surveys of Plant Cell and Molecular Biology*, Vol. 2, Miflin, B.J. (ed.). Oxford, pp. 253–317.
25. Varjonen, E., Vainio, E., Kalimo, K., Juntunen-Backman, K. & Savolainen, J. (1995) Skin-prick test and RAST responses to cereals in children with atopic dermatitis. Characterization of IgE-binding components in wheat and oats by an immunoblotting method. *Clin Exp Allergy* **25**, 1100.
26. Varjonen, E., Vainio, E. & Kalimo, K. (2000) Antigliadin IgE – indicator of wheat allergy in atopic dermatitis. *Allergy* **55**, 386.
27. Varjonen, E., Vainio, E. & Kalimo, K. (1997) Life-threatening, recurrent anaphylaxis caused by allergy to gliadin and exercise. *Clin Exp Allergy* **27**, 162.
28. Palosuo, K., Alenius, H., Varjonen, E. *et al*. (1999) A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J Allergy Clin Immunol* **103**, 912.
29. Tanabe, S., Arai, S., Yanagihara, Y., Takahashi, K. & Watanabe, M. (1996) A major wheat allergen has a Gln-Gln-Gln-Pro-Pro motif identified as an IgE-binding epitope. *Biochem Biophys Res Commun* **219**, 290.
30. Maruyama, N., Ichise, K., Katsube, T. *et al*. (1998) Identification of major wheat allergens by means of the *Escherichia* coli expression system. *Eur J Biochem* **255**, 739–745.
31. Gonzales, R., Polo, F., Zapatero, L., Caravaca, F. & Carreira, J. (1992) Purification and characterization of major inhalant allergens from soybean hulls. *Clin Exp Allergy* **22**, 748–755.
32. Codina, R., Lockey, R.F., Fernandez-Caldas, E. & Rama, R. (1997) Purification and characterization of a soybean hull allergen responsible for the Barcelona asthma outbreaks. II. Purification and sequencing of the Gly m 2 allergen. *Clin Exp Allergy* **27**, 424–430.
33. Odani, S., Koide, T., Ono, T., Seto, Y. & Tanaka, T. (1987) Soybean hydrophobic protein. *Eur J Biochem* **162**, 485–491.
34. Baud, F., Pebay-Peyroula, E., Cohen-Addad, C., Odani, S. & Lehmann, M.S. (1993) Crystal structure of hydrophobic protein from soybean; a member of a new cysteine-rich family. *J Mol Biol* **231**, 877–887.
35. Murzin, A.G., Brenner, S.E., Hubbard, T. & Chothia, C. (1995) SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* **247**, 536–540.
36. Shutov, A.D. & Bäumlein, H. (1999) Origin and Evolution of Seed Storage Globulins. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 543–561.
37. Dunwell, J.M. (1998) Cupins: a new superfamily of functionally diverse proteins that include germins and plant storage proteins. *Biotechnol Genet Eng Rev* **15**, 1–32.

38. Dunwell, J.M., Culham, A., Carter, C.E., Sosa-Aguirre, C.R. & Goodenough, P.W. (2001) Evolution of functional diversity in the cupin superfamily. *Trends Biochem Sci* **26**, 740–746.
39. Grzelczak, Z.F. & Lane, B.G. (1984) Signal resistance of a soluble protein to enzyme hydrolysis: an unorthodox approach to the isolation and purification of germin, a rare growth-related protein. *Can J Biochem Cell Biol* **62**, 1351–1353.
40. Lane, B.G., Dunwell, J.M., Ray, J.A., Schmitt, M.R. & Cuming, A.C. (1993) Germin, a marker of early plant development, is an oxalate oxidase. *J Biol Chem* **268**, 12239–12242.
41. Woo, E.J., Dunwell, J.M., Goodenough, P.W., Marvier, A.C. & Pickersgill, R.W. (2000) Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nat Struct Biol* **7**, 1036–1040.
42. Leitner, A., Jensen-Jarolim, E., Grimm, R. *et al.* (1998) Allergens in pepper and paprika. Immunological investigation of the celery–birch–mugwort–spice syndrome. *Allergy* **53**, 36–41.
43. Argos, P., Narayana, S.V.L. & Nielsen, N.C. (1985) Structural similarity between legumin and vicilin storage proteins from legumes. *EMBO J* **4**, 1111–1117.
44. Lawrence, M.C. (1999) Structural Relationships of 7S and 11S Globulins. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 517–541.
45. Lawrence, M.C., Suzuki, E., Varghese, J.N. *et al.* (1990) The three-dimensional structure of the seed storage protein phaseolin at 3Å resolution. *EMBO J*, **9**, 9–15.
46. Lawrence, M.C., Izard, T., Beuchat, M., Blagrove, R.J. & Colman, P.M. (1994) Structure of phaseolin at 2.2 Å resolution. Implications for a common vicilin/legumin structure and the genetic engineering of storage proteins. *J Mol Biol* **238**, 748–776.
47. Ko, T.-P., Ng, J.D. & McPherson, A. (1993) The three-dimensional structure of canavalin from jack bean (*Canavalia ensiformis*). *Plant Physiol* **101**, 729–744.
48. Ko, T.-P., Day, J. & McPherson, A. (2000) The refined structure of canavalin from jack bean in two crystal forms at 2.1 and 2.0 Å resolution. *Acta Crystallogr D Biol Crystallog* **56**, 411–420.
49. Maruyama, N., Adachi, M., Takahashi, K. *et al.* (2001) Crystal structures of recombinant and native soybean β-conglycinin β homotrimers. *Eur J Biochem* **268**, 3595–3604.
50. Adachi, M., Takenaka, Y., Gidamis, A.B., Mikami, B. & Utsumi, S. (2001) Crystal structure of soybean proglycinin A1aB1b homotrimer. *J Mol Biol* **12**, 291–305.
51. de Barros, E.G. & Larkins, B.A. (1990) Purification and characterization of zein-degrading proteases from endosperm of germinating maize seeds. *Plant Physiol* **94**, 297–303.
52. Mikkonen, A., Porali, I., Cercos, M. & Ho, T.-H.D. (1996) A major cysteine proteinase, EPB, in germinating barley seeds: structure of two intronless genes and regulation of expression. *Plant Mol Biol* **31**, 239–254.
53. Fischer, J., Becker, C., Hillmer, S. *et al.* (2000) The families of papain- and legumain-like cysteine proteinases from embryonic axes and cotyledons of *Vicia* seeds: developmental patterns, intracelular localization and functions in globulin proteolysis. *Plant Mol Biol* **43**, 83–101.
54. Becker, C., Fischer, J., Nong, V.H. & Müntz, K. (1994) PCR cloning and expression analysis of cDNAs encoding cysteine proteinases from germinating seeds of *Vicia sativa* L. *Plant Mol Biol* **26**, 1207–1212.
55. Jones, M.L., Larsen, P.B. & Woodson, W.R. (1995) Ethylene-regulated expression of a carnation cysteine proteinase during flower petal senescence. *Plant Mol Biol* **28**, 505–512.
56. Drake, R., John, I., Farrell, A., Cooper, W., Schuch, W. & Grierson, D. (1996) Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence. *Plant Mol Biol* **30**, 755–767.
57. Gietl, C., Wimmer, B., Adamec, J. & Kalousek, F. (1997) A cysteine endopeptidase isolated from castor bean endosperm microbodies processes the glyoxysomal malate dehydrongenase precursor protein. *Plant Physiol* **113**, 863–871.
58. Jiang, B.-H., Siregar, U., Willeford, K.O., Luthe, D.S. & Williams, W.P. (1995) Association of a 33-kD cysteine proteinase found in corn callus with the inhibition of fall armyworm larval growth. *Plant Physiol* **108**, 1631–1640.
59. Pechan, T., Ye, L., Chang, Y.-M. *et al.* (2000) A unique 22-kD cysteine proteinase accumulates in response to larval feeding in maize genotypes resistant to fall armyworm and other lepidoptera. *Plant Cell* **12**, 1031–1040.
60. Gall, H., Kalveram, K.-J., Forck, G. & Tümmers, U. (1990) Kreuzallergie zwischen Kiwi, Thiolproteinasen, Pollen und Nahrungsmitteln. *Allergologie* **13**, 447–451.
61. Gall, H., Kalveram, K.-J., Forck, G. & Sterry, W. (1994) Kiwi fruit allergy: a new birch pollen-associated food allergy. *J Allergy Clin Immunol* **94**, 70–76.
62. Pastorello, E.A., Pravettoni, V., Ispano, M. *et al.* (1996) Identification of the allergenic components of kiwi fruit and evaluation of their cross-reactivity with timothy and birch pollens. *J Allergy Clin Immunol* **98**, 601–610.
63. Pastorello, E.A., Conti, A., Pravettoni, V. *et al.* (1998) Identification of actinidin as the major allergen of kiwi fruit. *J Allergy Clin Immunol* **101**, 531–537.

64. Fahlbush, B., Rudeschko, O., Schumann, C. *et al*. (1998) Further characterisation of IgE-binding antigens in kiwi with particular emphasis on glycoprotein allergens. *J Investig Allergol Clin Immunol* **8**, 325–332.
65. Möller, M., Paschke, A., Vieluf, D., Kayma, M., Vieths, S. & Steinhart, H. (1997) Characterization of allergens in kiwi fruit and detection of cross-reactivities with allergens of birch pollen and related fruits. *Food Agric Immunol* **9**, 107–121.
66. Baur, X. (1979) Studies on the specificity of human IgE-antibodies to the plant proteases papain and bromelain. *Clin Allergy* **9**, 451–457.
67. Diez-Gomez, M.L., Quirce, S., Aragoneses, E. & Cuevas, M. (1998) Asthma caused by *Ficus benjamina* latex: evidence of cross-reactivity with fig fruit and papain. *Ann Allergy Asthma Immunol* **80**, 24–30.
68. Zentner, A., Jeep, S., Wahl, R., Kunkel, G. & Kleine-Tebbe, J. (1997) Multiple IgE-mediated sensitizations to enzymes after occupational exposure: evaluation by skin prick test, RAST, and immunoblot. *Allergy* **52**, 928–934.
69. Soto-Mera, M.T., Lopez-Rico, M.R., Filgueira, J.F., Villamil, E. & Cidras, R. (2000) Occupational allergy to papain. *Allergy* **55**, 983–984.
70. Blanco, C., Ortega, N., Castillo, R., Alvarez, M., Dumpierrez, A.G. & Carrillo, T. (1998) *Carica papaya* pollen allergy. *Ann Allergy Asthma Immunol* **81**, 171–175.
71. Kalinski, A., Melroy, D.L., Dwivedi, R.S. & Herman, E.M. (1992) A soybean vacuolar protein (P34) related to thiol proteases is synthesized as a glycoprotein precursor during seed maturation. *J Biol Chem* **267**, 12068–12076.
72. Ogawa, T., Tsuji, H., Bando, N. *et al*. (1993) Identification of the soybean allergenic protein, *Gly m* Bd 30K, with the soybean seed 34-kDa oil-body. *Biosci Biotechnol Biochem* **57**, 1030–1033.
73. Ji, C., Boyd, C., Slaymaker, D. *et al*. (1998) Characterization of a 34-kDa soybean binding protein for the syringolide elicitors. *PNAS* **95**, 3306–3311.
74. Baker, E.N. (1980) Structure of actinidin after refinement at 1.7 Å resolution. *J Mol Biol* **141**, 441–484.
75. Kamphuis, I.G., Kalk, K.H., Swarte, M.B. & Drenth, J. (1984) Structure of papain refined at 1.65 Å resolution. *J Mol Biol* **179**, 233–256.
76. Sumner, I.G., Harris, G.W., Taylor, M.A., Pickersgill, R.W., Owen, A.J. & Goodenough, P.W. (1993) Factors effecting the thermostability of cysteine proteinases from *Carica papaya*. *Eur J Biochem* **214**, 129–134.
77. Groves, M.R., Taylor, M.A., Scott, M., Cummings, N.J., Pickersgill, R.W. & Jenkins, J.A. (1996) The prosequence of procaricain forms an alpha-helical domain that prevents access to the substrate-binding cleft. *Structure* **15**, 1193–1203.
78. Coulombe, R., Grochulski, P., Sivaraman, J., Menard, R., Mort, J.S. & Cygler, M. (1996) Structure of human procathepsin L reveals the molecular basis of inhibition by the prosegment. *EMBO J* **15**, 5492–5503.
79. Carbonero, P. & García-Olmedo, F. (1999) A multigene family of trypsin α-amylase inhibitors from cereals. In: *Seed Proteins*, Shewry, P.R. & Casey, R. Kluwer Academic Publishers, Dordrecht, pp. 617–633.
80. Douliez, J.-P., Michon, T., Elmorjani, K. & Marion, D. (2000) Structure, biological and technological function of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J Cereal Sci* **32**, 1–20.
81. Woo, Y.-M., Hu, D.W.-N., Larkins, B.A. & Jung, R. (2001) Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. *Plant Cell* **12**, 2297–2317.
82. José-Estanyol, M. & Puigdoménech, P. (2000) Plant cell wall glycoproteins and their genes. *Plant Physiol Biochem* **38**, 97–108.
83. Kasarda, D.D., Okita, T.W., Bernardin, J.E. *et al*. (1984) Nucleic acid (cDNA) and amino acid sequences of α-type gliadins from wheat (*Triticum aestivum*). *Proc Nat Acad Sci USA* **81**, 4712–4716.
84. Bartels, D., Altosaar, I., Harberd, N.P., Barker, R.F. & Thompson, R.D. (1986) Molecular analysis of γ-gliadin gene families at the complex *Gli-1* locus of bread wheat (*T. aestivum* L.). *Theor Appl Genet* **72**, 845–853.
85. Colot, V., Bartels, D., Thompson, R. & Flavell, R. (1989) Molecular characterization of an active wheat LMW glutenin gene and its relation to other wheat and barley prolamin genes. *Mol Gen Genet* **216**, 81–90.

# 3 The 2S Albumin Proteins

RAFAEL I. MONSALVE, MAYTE VILLALBA, MANUEL RICO,
PETER R. SHEWRY and ROSALÍA RODRÍGUEZ

## 3.1 Historical background

The term albumin has a long history but by 1924 had become restricted to proteins which are soluble in *pure* water and coagulable by heat [1]. In fact, Osborne listed four well-characterised types of seed albumin, leucosin in cereal seeds (wheat, rye, barley), legumelin and phaselin in different types of legume seed and ricin in castor bean. Subsequently the term albumin continued to be applied as a generic name for water-soluble proteins until the landmark study of Youle and Huang [2] which indicated the existence of a discrete type of 2S albumin storage protein. Youle and Huang [2] used sucrose density ultracentrifugation to compare the major protein components in seeds of 12 species of plants. These included two legumes (peanut, lupin) and one monocotyledonous species (*Yucca*, Liliaceae). The profiles obtained showed that all species contained major components with sedimentation coefficients ($S_{20w}$) of about 2 and 11 and that all except three also contained 7S components. The 2S components were water soluble (i.e. albumins) and were calculated to account for between 20% and 60% of the total seed protein, based on the areas under the sedimentation profiles. Amino acid analyses demonstrated that most of the 2S albumins were rich in cysteine, up to 13.1 mol% in the Brazil nut albumin which also contained 17.3 mol% methionine. This led to interest in exploiting the Brazil nut and other methionine-rich albumins in crop improvement programmes, as discussed briefly below.

Subsequent studies confirmed that the 2S albumins do indeed form a discrete group of seed storage proteins, although not all of the 2S albumins studied by Youle and Huang [2] have been studied in sufficient detail to confirm their relationships. However, wider comparisons also demonstrated that they form part of a large group of proteins called the prolamin superfamily. These include a range of low molecular mass sulphur-rich proteins, some of which are also water soluble (notably the α-amylase/trypsin inhibitors of cereal seeds). In addition, related sequences are present as specific domains within the major prolamin (alcohol soluble) storage proteins of cereal seeds. The prolamin superfamily is discussed in detail in Chapter 2 and individual groups of proteins in Chapters 4 (lipid transfer proteins) and 5 (α-amylase/trypsin inhibitors).

## 3.2 Botanical distribution

The Pfam database of plant protein families (http://www.sanger.ac.uk/Software/Pfam) [3] lists sequences of 2S albumins from 18 genera (from 11 families) of dicotyledonous plants and from three genera of Gymnosperms (*Pinus, Picea, Pseudotsuga*). However, despite the report of Youle and Huang [2] that 2S albumins are present in seeds of *Yucca* (Liliaceae), they have not yet been confirmed in any monocotyledonous species,

either by direct protein analysis or based on expressed sequence tag (EST) or genomic sequencing. Nevertheless, the presence of related proteins in spores of the ferns *Onoclea* and *Matteuccia* [4–7] demonstrates that their origin predates the separation of Spermatophytes (seed plants) from lower plants.

### 3.3 Biosynthesis, processing and deposition

The 2S albumins are typical seed storage proteins in that they are synthesised on the rough endoplasmic reticulum (ER) and transported into the lumen with the cleavage of an *N*-terminal signal sequence. Protein folding is assumed to occur within the ER lumen, with the eight conserved cysteine residues which are present in all 2S albumins forming identical patterns of four intra-chain disulphide bonds (as shown in Fig. 3.1 and discussed later). The proteins are subsequently transported via the Golgi apparatus to the vacuole where they accumulate to form protein bodies [8, 9]. Glycosylation has not been reported but proteolytic processing does occur within the vacuole. In the vast majority of 2S albumins this proteolysis results in a two subunit (heterodimeric) structure, with a small subunit containing cysteine residues 1 and 2 and a large subunit containing cysteine residues 3 to 8. These two subunits remain associated by two of the disulphide bonds that are formed in the ER. A typical heterodimeric 2S albumin is napin from oilseed rape (*Brassica napus*) and related brassicas (Fig. 3.1). The proteolytic processing generally occurs at the *C*-terminal side of asparagine residues [8, 10] and may lead to the loss of one or more short peptide sequences: an *N*-terminal prosequence, a *C*-terminal peptide and a linker peptide between the two subunits (see Fig. 3.1).

However, variant types of 2S albumin also occur. In particular, sunflower (*Helianthus annus*, Compositae) albumins are not cleaved into two subunits although an *N*-terminal prosequence may be removed (Fig. 3.1). Furthermore, in sunflower and castor bean (*Ricinus communis*, Euphorbiaceae) some albumins are synthesised as pairs, being encoded by a single mRNA which is translated to give a precursor protein which is then processed (presumably in the vacuole) to give either two single chain albumins (sunflower) or two heterodimeric albumins (castor bean) (Fig. 3.1). The 2S albumin of lupin (*Lupinus angustifolius*, Leguminosae) is also unusual in that it contains nine cysteine residues. The additional cysteine residue is present at position 45 in the large subunit and is readily blocked by *N*-ethylmaleimide. It is, therefore, thought to be present as a free sulphydryl group [11].

### 3.4 Polymorphism of 2S albumins

In common with other types of storage proteins, 2S albumin fractions do not consist of single proteins but are polymorphic mixtures of structurally related proteins encoded by small gene families. This has been studied in most detail for napins. Krebbers *et al.* [12] reported that *Arabidopsis* contains only four napin genes which are closely linked in tandem array. They also isolated and sequenced the whole gene family but comparison with the directly determined protein sequence indicated that

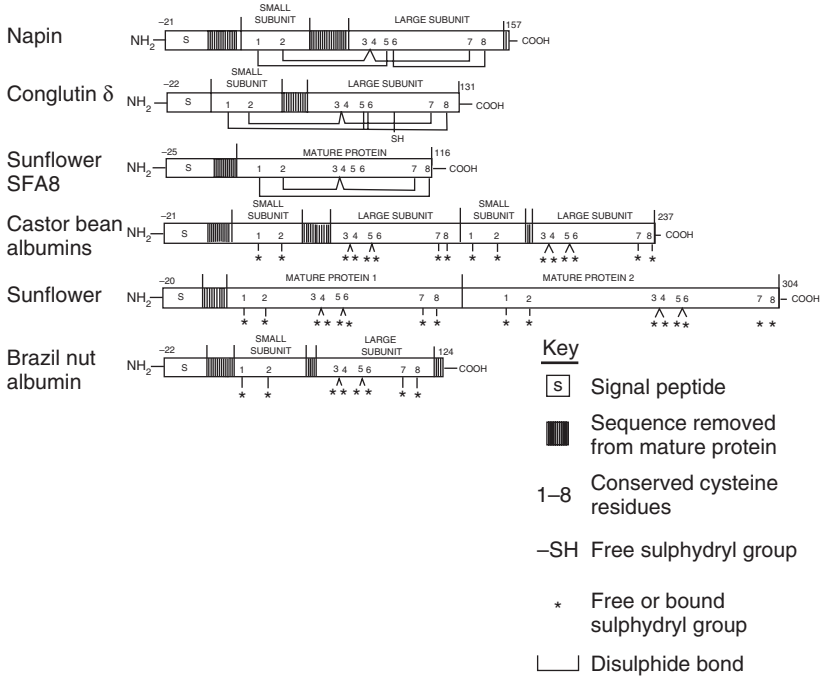


**Fig. 3.1** Schematic structures of typical and variant forms of 2S albumins. S indicates the signal peptide, hatched areas are sequences which are removed from the mature protein by proteolysis. 1–8 are conserved cysteine residues which have been shown to form the four disulphide bonds shown for some albumins. The disulphide bonds have not been mapped in the other albumins. The napin and conglutin δ are both synthesised as precursor proteins which are processed to give heterodimeric mature proteins, but differ in that conglutin δ has an additional *unpaired* cysteine residue (shown as SH). The sunflower albumins are single chain and are synthesised with either one (SFA8) or two mature albumins encoded by a single mRNA. The castor bean albumins are also synthesised as a pair encoded by a single mRNA but are processed to give two mature heterodimeric proteins. Based on sequences and alignments in Crouch *et al*. [95], Ericson *et al*. [96], Lilley & Inglis [11], Higgins *et al*. [22], Ampe *et al*. [18], Altenbach *et al*. [28], Gayler *et al*. [99], Irwin *et al*. [93], Kortt *et al*. [25], Egorov *et al*. [97] and Thoyts *et al*. [98]. Redrawn in part from Shewry & Pandya [100] with kind permission of Cambridge University Press.

only one of the genes was responsible for most of the protein synthesised. A detailed study of the napin proteins present in *Brassica* species (*B. rapa, B. oleracea* and *B. napus*) was reported by Monsalve and Rodríguez [13] with a total of 11 proteins being purified and characterised with respect to their amino acid compositions and masses by SDS-PAGE. The same group subsequently purified nine albumins from two species of radish (*Raphanus sativus, R. raphanistrum*) [14]. Southern blot analyses have been reported to show about 10 [15] and over 16 [16] napin genes per haploid genome of *B. napus* (which is an amphidiploid hybrid between *B. oleracea* and *B. rapa*). Similarly, Raynal *et al*. [17] characterised cDNAs representing two subfamilies of napins and estimated the presence of a minimum of six genes.

Other species have been studied in less detail but at least six forms of the Brazil nut albumin are present [18] while sunflower albumins can be separated into about 11 to 13 components by electrophoresis and RP-HPLC [19, 20].

### 3.5 Amino acid compositions

2S albumins tend to be rich in glutamine and arginine, amino acids which contain two and three nitrogen atoms, respectively. Hence they provide efficient storage of nitrogen. However, of more interest is their high contents of sulphur-containing amino acids, cysteine and methionine. All contain eight conserved cysteine residues and hence have higher proportions of cysteine than most other proteins. Specific cysteine-rich forms have also been reported in quinoa (15.6 mol%) [21] and pea (11.2 mol%) [22], but the full amino acid sequence of the quinoa protein is not yet available and its precise relationship to other 2S albumins remains to be determined while the sequence of the pea albumin is only distantly related, if at all, to those of the *typical* 2S albumins such as napins. Methionine-rich 2S albumins also occur in Brazil nut and relatives [2, 18, 23, 24], sunflower [2, 19, 25], cottonseed [26] and amaranthus [27].

Brazil nut albumins account for about 30% of the proteins extracted with buffers containing salt or SDS and contain about 19 mol% Met and 8 mol% Cys [23]. They comprise at least six different forms, with the large subunits containing 14 or 15 Met residues [18, 28]. The mature heterodimeric albumin comprises subunits of $M_r$ about 3000 and 9000 which are released from an $M_r$ 18 000 precursor protein by three stepwise cleavages [18, 28].

In contrast to Brazil nut, only two of the 11 to 13 albumins present in sunflower seeds are rich in methionine, SFA7 and SFA8. These proteins appear to have similar structures and sequences [29] but only SFA8, which is usually the major component of the two, has been studied in detail. SFA8 is a single chain albumin of 103 residues including 16 methionines and is synthesised as a precursor with a 13 residue pro-sequence [25].

The Brazil nut albumin and SFA8 have both been expressed in seeds of other species in order to confer increased levels of methionine [30–34]. However, further development of lines expressing the Brazil nut protein has been halted following the demonstration that it is a major allergen (see below) [35, 36]. Similarly, the recent demonstration that SFA8 may also be allergenic [37, 38] will presumably also limit commercial development of lines expressing this protein.

### 3.6 Amino acid sequences

The most widely studied 2S albumins are the napins of *B. napus* and related *napin-like* proteins from other brassicas. A vast number of sequences have been reported for these proteins, as summarised in Table 3.1 which lists nucleotide sequences identified as having significant similarity to the yellow mustard allergen, Sin a 1, by a BLAST search. Alignments of the sequences of single albumins from each of the nine species are shown in Fig. 3.2. It should be noted that these species are restricted to the Brassicaceae and, with the exception of the *Arabidopsis* sequences, the similarities range from 85% to 95%.

An alignment of the amino acid sequences of 2S albumins from a wider range of species is shown in Fig. 3.3, although this is restricted to proteins which are known to

**Table 3.1** Details of 2S albumin sequences identified using a BLAST search of the GenBank + EMBL + DDBJ + PDB databases using the Sin a 1 gene of *Sinapis alba* (yellow mustard) as a query. The sequences of the single accessions listed for each species are aligned in Fig. 3.2. Percentages of identity and similarity are relative to the Sin a 1 (*Sinapis alba*) gene which is shorter than the other sequences

| Accession number | Species | Number of sequences in BLAST search | % of identity (% of similarity) |
|---|---|---|---|
| X17542 | *Brassica napus* | 10 | 92.4 (94.5) |
| M64631 | *B. campestris* | 4 | 87.6 (92.4) |
| X65039 | *B. nigra* | 2 | 86.9 (89.7) |
| X65040 | *B. juncea* | 2 | 84.1 (89.0) |
| X65038 | *B. oleracea* | 2 | 85.5 (90.3) |
| X74813 | *B. carinata* | 1 | 87.6 (90.0) |
| A25773 | *Raphanus sativus* | 4 | 80.7 (84.4) |
| AF3370541 | *Arabidopsis thaliana* | 11 | 54.5 (62.8) |
| X91799 | *Sinapis alba* | 6 | used as reference |

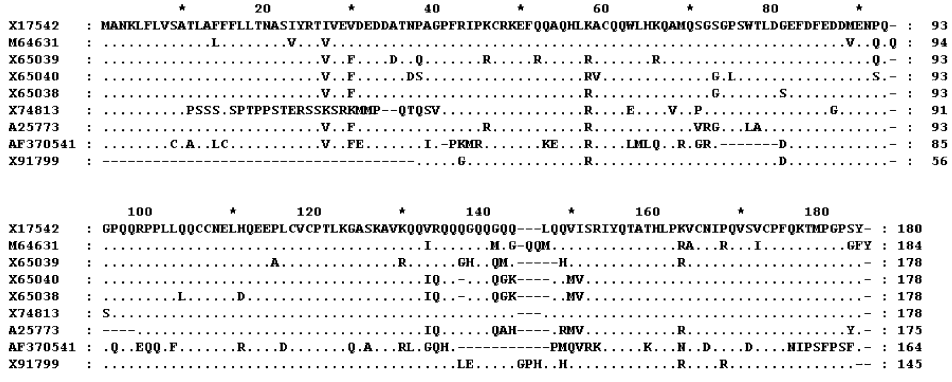


**Fig. 3.2** Sequence alignment of napin-like protein genes after a BLAST [101] search with the nucleotide sequence of the *Sinapis alba* (yellow mustard) Sin a 1 gene (accession number X91799) used as query. Accession numbers of EMBL/GenBank/DDJB databases are shown before each sequence with details provided in Table 3.1. Translated sequences of the corresponding genes were used for the alignment, with differences relative to the top sequence being shown. Dashes correspond to gaps opened by ClustalW program [102] for best alignment; in the case of the Sin a 1 gene the sequence starts with the mature protein, while in all the other cases the complete coding sequences are shown.

be allergenic (from brassicas, castor bean, Brazil nut, sunflower, peanut, walnut and cottonseed).

It can be seen that these proteins share the conserved pattern of eight cysteine residues (–C–C–CC–CXC–C–C–) that is characteristic of members of the prolamin superfamily (see Chapter 2) and information currently available shows that these cysteines form a very similar pattern of inter- and intra-chain disulphide bonds. However, the primary structures may show low levels of sequence identity, below 15% in some cases. This results from divergence in the nucleotide sequences combined with differential processing of precursor proteins between species (as discussed above).

Another general feature of 2S albumins is the presence of a *hypervariable* region present in the large chain (or in the corresponding region of single chain albumins).

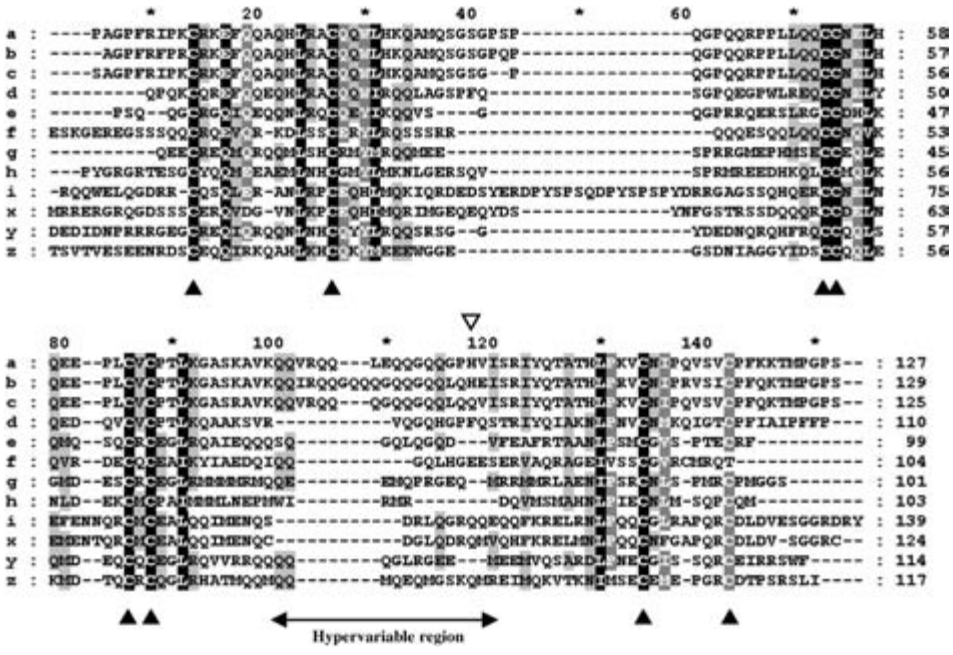


**Fig. 3.3** Multiple sequence alignments of allergenic 2S albumins. (a) *Sinapis alba* Sin a 1 [59]; (b) *Brassica juncea* Bra j 1 [39]; (c) *Brassica napus* Bra n 1 [71]; (d) *B. napus* BnIb [91, 92]; (e) *Ricinus communis* Ric c 1 [72, 93]; (f) *R communis* Ric c 3 [72, 93]; (g) *Bertholletia excelsa* Ber e 1 [18, 36]; (h) *Helianthus annuus* SFA8 [25, 38]; (i) *Arachis hypogea* Ara h 2 [70]; (x) *A. hypogea* Ara h 6 [94]; (y) *Juglans regia* Jug r 1 [58]; (z) *Gossypium hirsutum* Mat5-DC [26, 55]. Sequences of the mature proteins (generally small and large chains together) were used for the alignment, except for sequences x, y and z, for which only DNA data are known (the deduced amino acid sequences were trimmed at the *N*-terminal ends to fit the length of the longest mature protein). Solid triangles show the consensus cysteine pattern of 2S albumins and the open triangle shows a specific position discussed in the text. Numbers on the right of the alignment are residue numbers. The ClustalW program [102] was used with dashes representing gaps introduced for best alignment. Conserved residues are shaded with darker shading representing higher conservation. Reprinted with permission of the publisher from Monsalve *et al*. [65].

This is indicated in Fig. 3.3. Structural studies (see below) indicate that this sequence forms a variable loop which is an important antigenic region [39, 40].

## 3.7 Biological role

The most important biological role of the 2S albumins is undoubtedly storage, providing reserves of nitrogen, carbon and sulphur to support germination and seedling growth. However, several types of biological activity have also been reported for specific albumins leading to the suggestion that they may have other biological roles. Terras *et al*. [41] reported that 2S albumins from seeds of radish (*Raphanus sativus*) inhibited the growth of plant pathogenic fungi, although this activity was antagonised by cations ($K^+$, $Mg^{2+}$). Subsequent studies showed that the activity was enhanced in the presence of wheat or barley thionins (by two- or three-fold) and that the growth of Gram-positive

bacteria was also inhibited [42]. This activity appears to be due to effects on membranes resulting in leakage of components from cells and is consistent with a role in protection against pathogenic micro-organisms. Similarly, the demonstration that napins and *napin-like* proteins from kohlrabi (*B. napus* var *rapifera*), charlock (*Sinapis arvensis*) and black mustard (*B. nigra*) are inhibitory to serine proteinases (trypsin, subtilisin and, in the case of charlock, also α-chymotrypsin) is also consistent with a role in defence [43–45].

Finally, a role in regulating seed germination has been proposed based on the demonstration that the reduced chains of radish (*Raphanus sativus*) albumins act as calmodulin antagonists, inhibiting calmodulin-dependant kinase activities [46–48].

### 3.8 Three-dimensional structures

The three-dimensional structure of a napin (BnIb) purified from seeds of *B. napus* (oilseed rape) was determined by NMR spectroscopy [40]. This protein, which corresponds to sequence d in Fig. 3.3, has a lower molecular mass than the typical napins from this species and is also less heterogenous. The structure showed a compact conformation with a high proportion of α-helical structure (Plate 3.1(a)). This is consistent with some data from circular dichroism spectroscopy [14, 49–51], but other authors have reported the presence of β-sheet structure [43, 44].

More recently, NMR spectroscopy has been used to determine the structures of two recombinant 2S albumins (i.e. expressed in heterologous host systems) [52]. These are the unprocessed precursors of BnIb (pro BnIb) (Plate 3.1(b)) and the castor bean albumin Ric c 3 (Plate 3.1(c)). Preliminary data have also been obtained on the methionine-rich sunflower albumin SFA8, using protein purified from seeds [52].

All of the proteins, including SFA8, appear to have similar structures, comprising five α-helices arranged in a right-handed superhelix. Comparison of the napin (Plate 3.1(a)) and pronapin (Plate 3.1(b)) structures shows that the connection between the small (shown in blue) and large (shown in green) chains is cleaved post-translationally with the loss of part of the connecting loop (shown in black in Plate 3.1(b)).

Heterogeneity in the sample precluded the determination of a high resolution structure of the BnIb napin and only the global fold could be determined (Plate 3.1(a)). Although still under refinement the pro BnIb structure (Plate 3.1(b)) provides improved definition of the helical components while the castor bean Ric c 3 structure is the best defined 2S albumin structure available at present. It can be seen that the precise orientations of the five helices show some differences between the three proteins, with the pro BnIb being more similar to Ric c 3 than to the mature BnIb.

The three-dimensional structures of 2S albumins are similar to those of related small sulphur-rich proteins of the prolamin superfamily, non-specific lipid transfer proteins (ns LTPs) (Plate 3.1(d)), α-amylase/trypsin inhibitors (Plate 3.1(e)) and soybean hydrophobic protein (Plate 3.1(f)). These proteins are discussed in more detail in other chapters in this volume.

The high level of sequence identity between Sin a 1 and BnIb allows the atomic coordinates determined for BnIb to be used to construct a molecular model for the three-dimensional structure of the Sin a 1 protein. This shows that the greatest

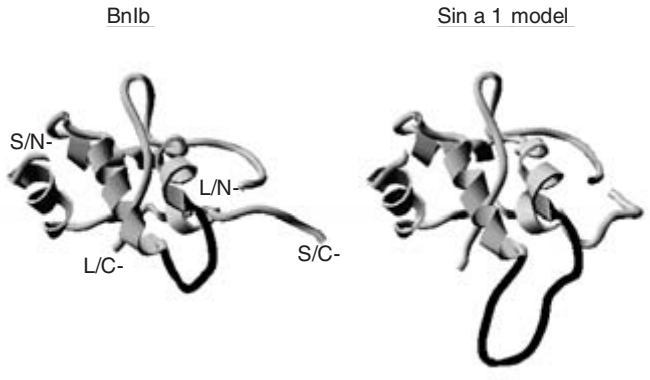


**Fig. 3.4** Schematic ribbon representations of the napin BnIb (PDB code: 1PNB) [40] structure and the Sin a 1 model [59]. The loop that corresponds to the hypervariable region is drawn in black. The termini of the small and large chains of BnIb are labelled as S/N- and L/N- for the *N*-terminal, respectively, and S/C- and L/C- for the *C*-terminal, respectively. Reprinted with permission of the publisher from Monsalve *et al*. [65].

difference between the two proteins lies in the variable loop region, which is especially short in BnIb (Fig. 3.4). This difference is also apparent in the sequence alignment shown in Fig. 3.3

The structure of the methionine-rich sunflower albumin SFA8 has been modelled, using the α-amylase/trypsin inhibitor from ragi (Indian finger millet) (PDB code 1B1U) as a template. The model predicts the presence of a hydrophobic face which is consistent with the good emulsifying properties of the protein in oil/water mixtures [53]. Completion of the three-dimensional structure currently being determined by NMR spectroscopy will confirm the validity of the modelling approach.

## 3.9 Allergenicity of 2S albumins

The 2S albumins constitute a major group of plant food allergens. Early studies reported allergenic components in peas [54], cotton seeds [55], soybean [56] and castor bean [57] while Table 3.2 lists 12 components, from nine species, which have been characterised over the last two decades. Teuber *et al*. [58] have suggested that the 2S albumins constitute an intrinsically allergenic family of proteins.

The allergenic 2S albumins that have been studied in most detail are Sin a 1 [59, 60] and Bra j 1 [39, 61], which are the major allergens of yellow (*Sinapis alba*) and oriental (*Brassica juncea*) mustards, respectively. Mustard has been reported to provoke very strong atopic reactions, sometimes leading to anaphylactic shock which requires urgent medical care [59, 62–65]. The allergens responsible for these reactions were isolated and structurally characterised [49, 59, 61]. In the case of Sin a 1, B-cell epitopes were mapped by testing the reactivity of ten monoclonal antibodies in competition and complementation assays, as well as testing their recognition towards the chemically modified allergen [60]. Two immunodominant regions were defined,

**Table 3.2** Characteristics of 2S albumins that have been defined as allergens. Data based on sequences of mature proteins are included wherever possible, complemented with data on the percursors deduced from cDNAs when these are complete. Accession numbers correspond to SwissProt and TrEMBL databases, except for L77197 that corresponds to Genbank. Proteins are shown in the same order as in Fig. 3.3. Reprinted, with permission of the publisher, from Monsalve *et al*. [65]

| Allergen name | Species and common name | References | Database accession number | Precursor length (*) | Mature chains' size (**) | MW of mature protein (Da) | Ident. % (Simil. %) |
|---|---|---|---|---|---|---|---|
| Sin a 1 | *Sinapis alba* (yellow mustard) | [59] | P15322 | 145 | 39/88 | 14180 | (***) |
| Bra j 1 | *Brassica juncea* (oriental mustard) | [39] | P80207 | | 37/92 | 14644 | 86 (91) |
| Bra n 3 | *Brassica napus* (rapeseed) | [71] | P80208 | | 37/88 | 14035 | 92 (94) |
| BnIb | *Brassica napus* (rapeseed) | [91, 92, 89] | P24565 | | 31/79 | 12691 | 47 (61) |
| Ric c 1 | *Ricinus communis* (castor bean) | [72, 93] | P01089 | 258 (*) | 34/65 | 11212 | 25 (43) |
| Ric c 3 | *Ricinus communis* (castor bean) | [72, 93] | P01089 | 258 (*) | 37/70 | 12032 | 20 (35) |
| Ber e 1 | *Bertholletia excelsa* (Brazil nut) | [18, 36] | P04403 | 146 | 28/73 | 12218 | 18 (39) |
| SFA8 | *Helianthus annuus* (common sunflower) | [25, 38] | P23110 | 141 | 103 | 12155 | 14 (28) |
| Ara h 2 | *Arachis hypogaea* (peanut) | [70] | L77197 | 157 | 138 | 16637 | 14 (31) |
| Ara h 6 | *Arachis hypogaea* (peanut) | [94] | Q9SQG5 | 129 | | | 17 (34) |
| Jug r 1 | *Juglans regia* (English walnut) | [58] | P93198 | 139 | | | 21 (38) |
| Mat5-D | *Gossypium hirsutum* (upland cotton) | [26, 55] | Q39787 | 139 | (27/76) | | 12 (31) |

(*) Size expressed as number of amino acids (only shown when the complete precursor is known). Ric c 1 and Ric c 3 are encoded by the same precursor.
(**) Defined as the number of amino acids of the small and large chains. In the case of SFA8 and Ara h 2, the mature protein is a single chain. For Mat5-D, the predicted size of chains is shown.
(***) Identity (Ident.%) and similarity (Simil.%) percentages correspond to the alignment shown in Fig. 3.3, and are referred to the pairwise comparison with Sin a 1. Identities correspond to exact matches between the proteins and similarities to conservative changes between them.

one of which was located very close to the *hypervariable region* of these 2S albumins. The latter was defined as a mustard-specific epitope [39] which was not present in napins of oilseed rape and is indicated by the open triangle in Fig. 3.3.

Detailed clinical studies of allergies caused by 2S albumins from mustard seeds have been reported only in recent years [66–68]. These studies showed an increasing incidence of mustard allergy in French children, and noted the importance of using new tests for the diagnosis of this type of allergy. In order to avoid strong reactions in oral food challenges a *labial food challenge* test was used to allow the clinical demonstration of allergenicity. The authors include mustard among the five most important food allergens (together with eggs, peanuts, cow's milk and cod) which were responsible for 78% of the cases of food allergy in 722 patients.

In the last five years, several cases of allergenicity due to other 2S albumins have been reported. In 1996, the methionine-rich 2S albumin from Brazil nut was introduced into soybean seeds to improve its nutritional quality for livestock feed, but allergenicity of the transgenic seed was reported [36]. Subsequently, an immunochemical characterisation of the major allergen, Ber e 1, was carried out [69]. In 1997, Ara h 2, a 2S albumin from peanut, was characterised as a major allergen affecting more than 85% of peanut-sensitive patients, and its immunodominant IgE epitopes were identified [70]. More recently, other allergenic 2S albumins have also been described and partially characterised, including Bra n 3 [71], Ric c 1 and Ric c 3 [72], Jug r 1 [58], SFA8 from sunflower [37, 38], as well as albumins from sesame seeds [73].

### 3.10 Structure/allergenicity relationships

The first three-dimensional data available for 2S albumins [40] revealed that these proteins had structural similarity with other plant proteins: the hydrophobic protein from soybean (HPS) [74] and the non-specific lipid transfer proteins (LTPs) [75–77]. The same global fold is also present in the cereal α-amylase/trypsin inhibitors [78], and these three groups of plant proteins constitute the *all-alpha* protein class, according to the classification of the SCOP structural database [79]. They are described as *disulphide-rich, with a common fold of four helices, folded leaf, right-handed superhelix*. This structural similarity is of importance if we consider that some components of all the protein groups mentioned exhibit allergenic properties. The primary structures of these groups of proteins differ significantly and it is not possible to align their sequences, unless knowledge of their spatial organisation is used to force the matching of the cysteines that form conserved disulphide bonds [40, 51]. These structural similarities have led several authors [40, 51, 78] to suggest the existence of a common ancestral protein in plants that has diverged to give proteins with different functional activities, but with a conserved common conformation.

An important structural feature of 2S albumins in relation to allergenicity could be their compactness resulting from their disulphide bond arrangement. This would confer unusually high stability both to thermal denaturation and to digestion by proteolytic enzymes. Indeed, this is the case for 2S albumins [80–82] and for LTPs [83, 84]. Another important property could be the ability of these proteins to interact with membranes, as reported for LTPs [85] and 2S albumins [81]. Both of these properties may underlie their properties as food allergens since resistance to digestion and interaction with membranes are key factors for the allergenicity of food components [81, 82, 86]. These properties would allow the proteins to reach the gastrointestinal tract, almost intact, and would also favour increased cellular uptake, reduced neutralisation by secretory antibodies and decreased degradation in the blood stream [81].

In conclusion, 2S albumins constitute a family of important food allergens which are structurally related to other plant proteins but still need to be studied in more detail in order to completely elucidate their antigenic and allergenic structure. Several 2S albumins are being studied at the moment in order to produce recombinant forms [87–90]. These will allow more detailed studies to be made, leading to improved applications in the diagnosis and immunotherapy of this type of food allergy.

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## References

1. Osborne, T.B. (1924) *The Vegetable Proteins*. Longmans, Green & Co., London.
2. Youle, R.J. & Huang, A.H.C. (1981) Occurrence of low molecular weight and high cysteine containing albumin storage proteins in oilseeds of diverse species. *Am J Bot* **68**, 44–48.
3. Bateman, A., Birney, E., Cerruti, L. *et al*. (2002) The Pfam protein families database. *Nucleic Acids Res* **30**, 276–280.
4. Templeman, T.S., DeMaggio, A.E. & Stetler, D.A. (1987) Biochemistry of fern spore germination: globulin storage proteins in *Matteuccia struthiopteris* L. *Plant Physiol* **85**, 343–349.
5. Templeman, T.S., Stein, D.B. & DeMaggio, A.E. (1988) A fern spore storage protein is genetically similar to the 1.7S seed storage protein of *Brassica napus*. *Biochem Genet* **26**, 595–603.
6. Templeman, T.S. & DeMaggio, A.E. (1990) Biochemistry of fern spore proteins: globulin storage proteins in *Onoclea sensibilis* and *Osmunda cinnamomea*. *Am J Bot* **77**, 284–287.
7. Rödin, J. & Rask, L. (1990) Characterization of matteuccin, the 2.2S storage protein of the ostrich fern. Evolutionary relationship to angiosperm seed storage proteins. *Eur J Biochem* **192**, 101–107.
8. Hara-Hishimura, I., Takeuchi, Y., Inoue, I. & Nishimura, M. (1993) Vesicle transport and processing of the precursor to 2S albumin in pumpkin. *Plant J* **4**, 793–800.
9. Scarafoni, A., Carzaniga, R., Harris, N. & Croy, R.R.D. (2001) Manipulation of the napin primary structure alters its packaging and deposition in transgenic tobacco (*Nicotiana tabacum* L.) seeds. *Plant Mol Biol* **46**, 727–739.
10. D'Hondt, K., Bosch, D., Van Damme, J., Goethals, M., Vandekerckhove, J. & Krebbers, E. (1993) An aspartic proteinase present in seeds cleaves *Arabidopsis* 2S albumin precursors *in vitro*. *J Biol Chem* **268**, 20884–20891.
11. Lilley, G.G. & Inglis, A.S. (1986) Amino acid sequence of conglutin δ, a sulfur-rich seed protein of *Lupinus angustifolius* L. Sequence homology with the C-III α-amylase inhibitor from wheat. *FEBS Lett* **195**, 235–241.
12. Krebbers, E., Herdies, L., De Clercq, A. *et al*. (1988) Determination of the processing sites of an *Arabidopsis* 2S albumin and characterization of the complete gene family. *Plant Physiol* **87**, 859–866.
13. Monsalve, R.I. & Rodríguez, R. (1990) Purification and characterization of proteins from the 2S fraction from seeds of the Brassicaceae family. *J Exp Bot* **41**, 89–94.
14. Monsalve, R.I., Menéndez-Arias, L., González de la Peña, M.A., Batanero, E., Villalba, M. & Rodríguez, R. (1994) Purification and characterization of napin-like proteins from radish. *J Exp Bot* **45**, 1169–1176.
15. Josefsson, L.-G., Lenman, M., Ericson, M.L. & Rask, L. (1987) Structure of a gene encoding the 1.7 S storage protein, napin, from *Brassica napus*. *J Biol Chem* **262**, 12196–12201.
16. Scofield, S.T. & Crouch, M.L. (1987) Nucleotide sequence of a member of the napin storage protein family from *Brassica napus*. *J Biol Chem* **262**, 12202–12208.
17. Raynal, M., Depigny, D., Grellet, F. & Delseny, M. (1991) Characterization and evolution of napin-encoding genes in radish and related crucifers. *Gene* **99**, 77–86.
18. Ampe, C., Van Damme, J., de Castro, L.A., Sampaio, M.J., Van Montagu, M. & Vandekerckhove, J. (1986) The amino-acid sequence of the 2S sulphur-rich proteins from seeds of Brazil nut (*Bertholletia excelsa* H.B.K.). *Eur J Biochem* **159**, 597–604.
19. Kortt, A.A. & Caldwell, J.B. (1990) Low molecular weight albumins from sunflower seed: identification of a methionine-rich albumin. *Phytochemistry* **29**, 2805–2810.
20. Anisimova, I.N., Fido, R.J., Tatham, A.S. & Shewry, P.R. (1995) Genotypic variation and polymorphism of 2S albumins of sunflower. *Euphytica* **83**, 15–23.

21. Brinegar, C. (1997) The seed storage proteins of quinoa. In: *Food Proteins and Lipids*, Damodaran, S. (ed.). Plenum Press, New York, pp. 109–115.
22. Higgins, T.J.V., Chandler, P.M., Randall, P.J. *et al*. (1986) Gene structure, protein structure, and regulation of the synthesis of a sulfur-rich protein in pea seeds. *J Biol Chem* **261**, 11124–11130.
23. Sun, S.S.M., Altenbach, S.B. & Leung, F.W. (1987) Properties, biosynthesis and processing of a sulfur-rich protein in Brazil nut (*Bertholletia excelsa* H.B.K.). *Eur J Biochem* **162**, 477–483.
24. Zuo, W.-N. & Sun, S.S.M. (1996) Purification and characterization of the methionine-rich 2S seed proteins from the Brazil nut family (*Lecythidaceae*). *J Agric Food Chem* **44**, 1206–1210.
25. Kortt, A.A., Caldwell, J.B., Lilley, G.G. & Higgins, T.J.V. (1991) Amino acid and cDNA sequences of a methionine-rich 2S protein from sunflower seed (*Helianthus annuus* L.). *Eur J Biochem* **195**, 329–334.
26. Galau, G.A., Wang, H.Y.-C. & Hughes, D.W. (1992) Cotton Mat5-A (C164) gene and Mat5-D cDNAs encoding methionine-rich 2S albumin storage proteins. *Plant Physiol* **99**, 779–782.
27. Segura-Nieto, M., Shewry, P.R. & Paredes-López, O. (1999) Globulins of the Pseudocereals: Amaranth, Quinoa, and Buckwheat. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Publishers, Dordrecht, pp. 453–475.
28. Altenbach, S.B., Pearson, K.W., Leung, F.W. & Sun, S.S.M. (1987) Cloning and sequence analysis of a cDNA encoding a Brazil nut protein exceptionally rich in methionine. *Plant Mol Biol* **8**, 239–250.
29. Anisimova, I.N., Konarev, Al.V., Gavrilova, V.A. *et al*. (2003) Polymorphism and inheritance of methionine-rich 2S albumins in sunflower. *Euphytica* **129**, 99–107.
30. Conceição, A.de S., Van Vliet, A. & Krebbers, E. (1994) Unexpectedly high expression levels of a chimeric 2S albumin seed protein transgene from a tandem array construct. *Plant Mol Biol* **26**, 1001–1005.
31. Altenbach, S.B., Pearson, K.W., Meecker, G., Staraci, L.C. & Sun, S.S.M. (1989) Enhancement of the methionine content of seed proteins by the expression of a chimeric gene encoding a methionine-rich protein in transgenic plants. *Plant Mol Biol* **13**, 513–522.
32. Altenbach, S.B., Kuo, C.-C., Staraci, L.C. *et al*. (1992) Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine. *Plant Mol Biol* **18**, 235–245.
33. Saalbach, I., Waddell, D., Pickardt, T., Schieder, O. & Müntz, K. (1995) Stable expression of the sulphur-rich 2S albumin gene in transgenic *Vicia narbonensis* increases the methionine content of seeds. *J Plant Physiol* **145**, 674–681.
34. Molvig, L., Tabe, L.M., Eggum, B.O. *et al*. (1997) Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. *Proc Nat Acad Sci USA* **94**, 8393–8398.
35. Melo, V.M.M., Xavier-Filho, J., Lima, M.S. & Prouvost-Danon, A. (1994) Allergenicity and tolerance to proteins from Brazil nut (*Bertholletia excelsa* H.B.K.). *Food Agric Immunol* **6**, 185–195.
36. Nordlee, J.A., Taylor, S.L., Townsend, J.A., Thomas, L.A. & Bush, R.K. (1996) Identification of a Brazil-nut allergen in transgenic soybeans. *N Engl J Med* **334**, 688–692.
37. Kelly, J.D., Hlywka, J.J. & Hefle, S.L. (2000) Identification of sunflower seed IgE-binding proteins. *Int Arch Allergy Immunol* **121**, 19–24.
38. Kelly, J.D. & Hefle, S.L. (2000) 2S methionine-rich protein (SSA) from sunflower seed is an IgE-binding protein. *Allergy* **55**, 556–559.
39. Monsalve, R.I., González de la Peña, M.A., Menéndez-Arias, L., López-Otín, C., Villalba, M. & Rodríguez, R. (1993) Characterization of a new oriental-mustard (*Brassica juncea*) allergen, Bra j IE: detection of an allergenic epitope. *Biochem J* **293**, 625–632.
40. Rico, M., Bruix, M., González, C., Monsalve, R.I. & Rodríguez, R. (1996) 1H NMR assignment and global fold of napin BnIb, a representative 2S albumin seed protein. *Biochemistry* **35**, 15672–15682.
41. Terras, F.R.G., Schoofs, H.M.E., De Bolle, M.F.C. *et al*. (1992) Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J Biol Chem* **267**, 15301–15309.
42. Terras, F.R.G., Schoofs, H.M.E., Thevissen, K. *et al*. (1993) Synergistic enhancement of the antifungal activity of wheat and barley thionins by radish and oilseed rape 2S albumins and by barley trypsin inhibitors. *Plant Physiol* **103**, 1311–1319.
43. Svendsen, I.B., Nicolova, D., Goshev, I. & Genov, N. (1989) Isolation and characterization of a trypsin inhibitor from the seeds of kohlrabi (*Brassica napus* var. rapifera) belonging to the napin family of storage proteins. *Carlsberg Res Commun* **54**, 231–239.
44. Svendsen, I., Nicolova, D., Goshev, I. & Genov, N. (1994) Primary structure, spectroscopic and inhibitory properties of a two-chain trypsin inhibitor from the seeds of charlock (*Sinapis arvensis* L.), a member of the napin protein family. *Int J Peptide and Protein Res* **43**, 425–430.
45. Genov, N., Goshev, I., Nikolova, D., Georgieva, D.N., Filippi, B. & Svendsen, I. (1997) A novel thermostable inhibitor of trypsin and subtilisin from the seeds of *Brassica nigra*: amino acid sequence, inhibitory and spectroscopic properties and thermostability. *Biochim Biophys Acta* **1341**, 157–164.

46. Polya, G.M., Chandra, S. & Condron, R. (1993) Purification and sequencing of radish seed calmodulin antagonists phosphorylated by calcium-dependent protein kinase. *Plant Physiol* **101**, 545–551.
47. Neumann, G.M., Condron, R., Thomas, I. & Polya, G.M. (1996) Purification and sequencing of multiple forms of *Brassica napus* seed napin small chains that are calmodulin antagonists and substrates for plant calcium-dependent protein kinase. *Biochim Biophys Acta* **1295**, 23–33.
48. Neumann, G.M., Condron, R., Thomas, I. & Polya, G.M. (1996) Purification and sequencing of multiple forms of *Brassica napus* seed napin large chains that are calmodulin antagonists and substrates for plant calcium-dependent protein kinase. *Biochim Biophys Acta* **1295**, 34–43.
49. Menéndez-Arias, L., Monsalve, R.I., Gavilanes, J.G. & Rodríguez, R. (1987) Molecular and spectroscopic characterization of a low molecular weight seed storage protein from yellow mustard (*Sinapis alba* L.). *Int J Biochem* **19**, 899–907.
50. Muren, E., Ek, B., Bjork, I. & Rask, L. (1996) Structural comparison of the precursor and the mature form of napin, the 2S storage protein in *Brassica napus*. *Eur J Biochem* **242**, 214–219.
51. Pandya, M.J., Sessions, R.B., Williams, P.B. *et al.* (2000) Structural characterization of a methionine-rich, emulsifying protein from sunflower seed. *Proteins* **38**, 341–349.
52. Pantoja-Uceda, D., Bruix, M., Santoro, J., Rico, M., Monsalve, R.I. & Villalba, M. (2002) Solution structure of allergenic 2S albumins. *Biochem Soc Trans* **30**, 919–924.
53. Burnett, G.R., Rigby, N.M., Mills, E.N.C. *et al.* (2002) Characterization of the emulsification properties of 2S albumins from sunflower seed. *J Colloid and Interface Sci* **247**, 177–185.
54. Malley, A., Baecher, L., Mackler, B. & Perlman, F. (1975) The isolation of allergens from the green pea. *J Allergy Clin Immunol* **56**, 282–290.
55. Youle, R.J. & Huang, A.H. (1979) Albumin storage protein and allergens in cottonseeds. *J Agric Food Chem* **27**, 500–503.
56. Shibasaki, M., Suzuki, S., Tajima, S., Nemoto, H. & Kuroume, T. (1980) Allergenicity of major component proteins of soybean. *Int Arch Allergy Appl Immunol* **61**, 441–448.
57. Sharief, F.S. & Li, S.S. (1982) Amino acid sequence of small and large subunits of seed storage protein from *Ricinus communis*. *J Biol Chem* **257**, 14753–14759.
58. Teuber, S.S., Dandekar, A.M., Peterson, W.R. & Sellers, C.L. (1988) Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *J Allergy Clin Immunol* **101**, 807–814.
59. Menéndez-Arias, L., Moneo, I., Domínguez, J. & Rodríguez, R. (1988) Primary structure of the major allergen of yellow mustard (*Sinapis alba* L.) seed, Sin a I. *Eur J Biochem* **177**, 159–166.
60. Menéndez-Arias, L., Domínguez, J., Moneo, I. & Rodríguez, R. (1990) Epitope mapping of the major allergen from yellow mustard seeds, Sin a I. *Mol Immunol* **27**, 143–150.
61. González de la Peña, M.A., Menéndez-Arias, L., Monsalve, R.I. & Rodríguez, R. (1991) Isolation and characterization of a major allergen from oriental mustard seeds, Bra j I. *Int Arch Allergy Appl Immunol* **96**, 263–270.
62. Panconesi, E., Sertoli, A., Fabbri, P., Giorgini, S. & Spallanzani, P. (1980) Anaphylactic shock from mustard after ingestion of pizza. *Contact Dermatitis* **6**, 294–295.
63. Stricker, W.E., Anorve-Lopez, E. & Reed, C.E. (1986) Food skin testing in patients with idiopathic anaphylaxis. *J Allergy Clin Immunol* **77**, 516–519.
64. Monreal, P., Botey, J., Pena, M., Marin, A. & Eseverri, J.L. (1992) Mustard allergy. Two anaphylactic reactions to ingestion of mustard sauce. *Ann Allergy* **69**, 317–320.
65. Monsalve, R.I., Villalba, M. & Rodríguez, R. (2001) Allergy to mustard seeds: the importance of 2S albumins as food allergens. *Internet Symposium Food Allergens* **3**, 57–69.
66. Rance, F. & Dutau, G. (1997) Labial food challenge in children with food allergy. *Pediatr Allergy Immunol* **8**, 41–44.
67. Rance, F., Kanny, G., Dutau, G. & Moneret-Vautrin, D.A. (1999) Food hypersensitivity in children: clinical aspects and distribution of allergens. *Pediatr Allergy Immunol* **10**, 33–38.
68. Rance, F., Dutau, G. & Abbal, M. (2000) Mustard allergy in children. *Allergy* **55**, 496–500.
69. Bartolomé, B., Méndez, J.D., Armentia, A., Vallverdu, A. & Palacios, R. (1997) Allergens from Brazil nut: immunochemical characterization. *Allergol Immunopathol* (Madr) **25**, 135–144.
70. Stanley, J.S., King, N., Burks, A.W. *et al.* (1997) Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. *Arch Biochem Biophysiol* **342**, 244–253.
71. Monsalve, R.I., González de la Peña, M.A., López-Otín, C. *et al.* (1997) Detection, isolation and complete amino acid sequence of an aeroallergenic protein from rapeseed flour. *Clin Exp Allergy* **27**, 833–841.
72. Bashir, M.E., Hubatsch, I., Leinenbach, H.P., Zeppezauer, M., Panzani, R.C. & Hussein, I.H. (1998) Ric c 1 and Ric c 3, the allergenic 2S albumin storage proteins of *Ricinus communis*: complete primary structures and phylogenetic relationships. *Int Arch Allergy Immunol* **115**, 73–82.

73. Pastorello, E.A., Varin, E., Farioli, L. *et al*. (2001) The major allergen of sesame seeds (*Sesamum indicum*) is a 2S albumin. *J Chromatogr* **756**, 85–93.
74. Baud, F., Pebay-Peyroula, E., Cohen-Addad, C., Odani, S. & Lehmann, M.S. (1993) Crystal structure of hydrophobic protein from soybean; a member of a new cysteine-rich family. *J Mol Biol* **231**, 877–887.
75. Gincel, E., Simorre, J.P., Caille, A., Marion, D., Ptak, M. & Vovelle, F. (1994) Three-dimensional structure in solution of a wheat lipid-transfer protein from multidimensional 1H-NMR data. A new folding for lipid carriers. *Eur J Biochem* **226**, 413–422.
76. Shin, D.H., Lee, J.Y., Hwang, K.Y., Kim, K.K. & Suh, S.W. (1995) High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. *Structure* **3**, 189–199.
77. Gomar, J., Petit, M.C., Sodano, P. *et al*. (1996) Solution structure and lipid binding of a nonspecific lipid transfer protein extracted from maize seeds. *Protein Sci* **5**, 565–577.
78. Behnke, C.A., Yee, V.C., Trong, I.L. *et al*. (1998) Structural determinants of the bifunctional corn Hageman factor inhibitor: X-ray crystal structure at 1.95′ resolution. *Biochemistry* **37**, 15277–15288.
79. Murzin, A.G., Brenner, S.E., Hubbard, T. & Chothia, C. (1995) SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* **247**, 536–540.
80. Domínguez, J., Cuevas, M., Ureña, V., Munoz, T. & Moneo, I. (1990) Purification and characterization of an allergen of mustard seed. *Ann Allergy* **64**, 352–357.
81. Oñaderra, M., Monsalve, R.I., Mancheño, J.M. *et al*. (1994) Food mustard allergen interaction with phospholipid vesicles. *Euro J Biochem* **225**, 609–615.
82. Astwood, J.D., Leach, J.N. & Fuchs, R.L. (1996) Stability of food allergens to digestion *in vitro*. *Nat Biotechnol* **14**, 1269–1273.
83. Asero, R., Mistrello, G., Roncarolo, D. *et al*. (2000) Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* **122**, 20–32.
84. Luckett, S., Sessions, R.B., Michaelson, L. *et al*. (2001) Amino acid sequence and molecular modelling of a lipid transfer protein from sunflower (*Helianthus annus* L.) seeds. *Protein and Peptide Letters* **8**, 241–248.
85. Wirtz, K.W. (1991) Phospholipid transfer proteins. *Annual Review of Biochemistry* **60**, 73–99.
86. Aalberse, R.C. (2000) Structural biology of allergens. *J Allergy Clin Immunol* **106**, 228–238.
87. González de la Peña, M.A., Monsalve, R.I., Batanero, E., Villalba, M. & Rodríguez, R. (1996) Expression in *Escherichia coli* of Sin a 1, the major allergen from mustard. *Eur J Biochem* **237**, 827–832.
88. Robotham, J.M., Teuber, S.S., Sathe, S.K. & Roux, K.H. (2002) Linear IgE epitope mapping of the English walnut (*Juglans regia*) major food allergen, Jug r 1. *J Allergy Clin Immunol* **109**, 143–149.
89. Palomares, O., Monsalve, R.I., Rodriguez, R. & Villalba, M. (2002) Recombinant pronapin precursor produced in *Pichia pastoris* displays structural and immunologic equivalent properties to its mature product isolated from rapeseed. *Eur J Biochem* **269**, 2538–2545.
90. Fernández-Tornero, C., Ramón A., Navarro, M.L., Varela, J. & Giménez-Gallego, G. (2002) Synthesis of proteins with disulfide bonds in *E. coli* using defined culture media. *Biotechniques* **32**, 1238–1242.
91. Monsalve, R.I., López-Otín, C., Villalba, M. & Rodríguez, R. (1991) A new distinct group of 2S albumins from rapeseed. Amino acid sequence of two low molecular weight napins. *FEBS Lett* **295**, 207–210.
92. Villalba, M., Monsalve, R.I., Batanero, E. & Ledesma, A. (2000) Cloning and expression of BnI, a 2S albumin from *Brassica napus* seeds with allergenic properties. *Allergy* **55**, 128 (abstract).
93. Irwin, S.D., Keen, J.N., Findlay, J.B. & Lord, J.M. (1990) The *Ricinus communis* 2S albumin precursor: a single preproprotein may be processed into two different heterodimeric storage proteins. *Molecular and General Genetics* **222**, 400–408.
94. Kleber-Janke, T., Crameri, R., Appenzeller, U., Schlaak, M. & Becker, W.M. (1999) Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int. Arch. Allergy Immunol* **119**, 265–274.
95. Crouch, M.L., Tenbarge, K.M., Simon, A.E. & Ferl, R. (1983) cDNA clones for *Brassica napus* seed storage proteins: evidence from nucleotide sequence analysis that both subunits of napin are cleaved from a precursor polypeptide. *J Molecular and Applied Genetics* **2**, 273–283.
96. Ericson, M.L., Rödin, J., Lenman, M., Glimelius, K., Josefsson, L-G. & Rask, L. (1986) Structure of the rapeseed 1.7 S storage protein, napin, and its precursor. *J Biol Chem* **261**, 14576–14581.
97. Egorov, T.A., Odintsova, T.I., Musolyamov, A.Kh., Fido, R., Tatham, A.S. & Shewry, P.R. (1996) Disulphide structure of a sunflower seed albumin: conserved and variant disulphide bonds in the cereal prolamin superfamily. *FEBS Lett* **396**, 285–288.
98. Thoyts, P.J.E., Napier, J.A., Millichip, M. *et al*. (1996) Characterization of a sunflower seed albumin which associates with oil bodies. *Plant Sci* **118**, 119–125.

99. Gayler, K.R., Kolivas, S., Macfarlane, A.J. *et al*. (1990) Biosynthesis, cDNA and amino acid sequences of a precursor of conglutin δ, a sulphur-rich protein from *Lupinus angustifolius*. *Plant Mol Biol* **15**, 879–893.
100. Shewry, P.R. & Pandya, M.J. (1999) The 2S Albumin Storage Proteins. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). Kluwer Academic Plublishers, Dordrecht, pp. 563–586.
101. Altschul, S.F., Madden, T.L., Schaffer, A.A. *et al*. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
102. Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
103. Strobl, S., Mühlhahn, P., Bernstein, R. *et al*. (1995) Determination of the three-dimensional structure of the bifunctional α-amylase/trypsin inhibitor from ragi seeds by NMR spectroscopy. *Biochemistry* **34**, 8281–8293.

# 4 Plant Lipid Transfer Proteins: Relationships between Allergenicity and Structural, Biological and Technological Properties

DIDIER MARION, JEAN-PAUL DOULIEZ, MARIE-FRANÇOISE GAUTIER and KHALIL ELMORJANI

## 4.1 Introduction

Lipids are acylated hydrophobic molecules that display many different key functions in the physiology of plants. As esters of long fatty acid and alcohol chains (i.e. waxes), and hydrophobic polyesters of hydroxy fatty acids (i.e. cutin and suberin), they form the protective tissues of plant organs against biotic and abiotic stresses. As triesters of glycerol (i.e. triglycerides), lipids are the storage form of carbon and energy. As phospholipids and glycolipids, they form, in association with proteins and sterols, the membranes of cells and organelles to fulfil the necessary compartmentalisation of metabolic cellular pathways. Lipids and their oxidised derivatives (e.g. phosphatidylinositol, lysophosphatidic acid, jasmonate) are also involved in many important cell signalling pathways. All these functions require cellular and extra-cellular lipid trafficking. In regard to the insolubility of these hydrophobic molecules in the aqueous cellular and extra-cellular compartments, lipids are transported by soluble macromolecules and supra-molecular structures, lipid-binding proteins and lipoproteins, respectively.

As part of a search for lipid-binding proteins involved in the intracellular trafficking of membrane lipids, a family of small hydrophilic proteins called non-specific lipid transfer proteins (nsLTP) was discovered about 30 years ago [1]. Despite intensive work on their structure and diversity at both the protein and the genetic levels, the precise biological role of these proteins remains uncertain, but it is probable that they are not involved with intracellular lipid trafficking but with the formation of protective hydrophobic cutin and/or suberin layers and the defence of plants against microbial pathogens. Furthermore, as highlighted in malting and brewing processes, these lipid-binding proteins can also play a significant role in the end uses of plant-derived foods. Therefore, plant nsLTPs fulfil key biological and technological functions that make their genes of interest to improve both the agronomic and technological properties of most crop plants. However, the recent discovery that nsLTPs and structurally related plant proteins could be pan-allergens of plant-derived foods has brought new insights into the application of plant biotechnology and plant breeding programmes devoted to this specific family of food proteins. Therefore, the aim of this review is to relate our present knowledge of the structural, physico-chemical and functional properties of nsLTPs with their allergenic properties.

## 4.2 NsLTPs: an ubiquitous multigenic family of plant proteins

Lipid transfer proteins are ubiquitous proteins in the plant kingdom that can enhance intermembrane transfer without lipid specificity. Consequently, they were called

non-specific lipid transfer proteins (nsLTP). Until now, two main families with different molecular masses have been identified with lipid binding and/or lipid transfer activities being demonstrated for one or several members. One family has a molecular weight of about 9 kDa and is referred as nsLTP1 (Fig. 4.1) and the other of 7 kDa as nsLTP2 (Fig. 4.2) [2–4]. Both families are multigenic, and more than 150 amino acid sequences are registered in the data banks (BLAST at http://www.ncbi.nlm.nih.gov) which have been mainly deduced from the corresponding DNA (i.e. cDNA or genes). These genes are expressed in all plants including monocots and dicots, and in all plant organs (e.g. seeds, fruits, leaves, roots, stems, flowers, pollen). In *Arabidopsis thaliana* more than 30 different putative nsLTP1 and nsLTP2 can be identified from BLAST at http://www.arabidopsis.org. In a single organ such as wheat seed, at least ten different *nsLTP* genes are expressed during development (Gautier, personal communication), and in the mature seed, at least two major nsLTP1s have been identified in the aleurone layer [5] and in the embryo [6].

NsLTP1 and nsLTP2 are both characterised by a conserved cysteine motif that is now considered as the nsLTP signature. All these cysteine residues are involved in intramolecular disulphide bonds whose connectivities are strictly conserved among nsLTP1s. With regard to nsLTP1, a mismatch at the Cys-X-Cys motif is observed in the cysteine pairing for nsLTP2 (Fig. 4.2) that could be related to slight differences in the overall fold [3]. Except for the cysteine signature, nsLTP1 and nsLTP2 display low or no sequence identities. Another general feature of the primary structure of nsLTPs is the absence of tryptophan residues. Comparison with different

```
NLTP_PYRCO    -ITCSQVSANLAPCINYVRSGGAVPPA-CCNGIKTINGLAKTTPDRQAACNCLKNLAGSVSGVNPGNAE
NLTP_MALDO    -ITCGQVTSSLAPCIGYVRSGGAVPPA-CCNGIRTINGLARTTADRQTACNCLKNLAGSISGVNPNNAA
NLT1_PRUDO    -ITCGQVSSNLAPCINYVKGGGAVPPA-CCNGIRNVNNLARTTADRRAACNCLKQLSGSIPGVNPNNAA
NLT1_PRUAR    -ITCGQVSSSLAPCIGYVRGGGAVPPA-CCNGIRNVNNLARTTPDRRTACNCLKQLSGSISGVNPNNAA
NLT1_PRUPE    -ITCGQVSSALAPCIPYVRGGGAVPPA-CCNGIRNVNNLARTTPDRQAACNCLKQLSASVPGVNPNNAA
NLTP_PRUAV    -LTCGQVSSNLAPCIAYVRGGGAVPPA-CCNGIRNINNLAKTTADRQTACNCLKQLSASVPGVNANNAA
NLTP_MAIZE    AISCGQVASAIAPCISYARGQGSGPSAGCCSGVRSLNNAARTTADRRAACNCLKNAAAGVSGLNAGNAA
NLTA_WHEAT    -IDCGHVDSLVRPCLSYVQGG-PGPSGQCCDGVKNLHNQARSQSDRQSACNCLKGIARGIHNLNEDNAR
NLT1_HORVU    -LNCGQVDSKMKPCLTYVQGG-PGPSGECCNGVRDLHNQAQSSGDR-TVCNCLKGIARGIHNLNLNNAA
NLT6_AMBAR    SPTCDTVQNILAPCAGFLTG--QEPSKACCTGVNNLNNSRKTKADRVAVCNCIKELTKSIA-YDPKRMP
NL11_PARJU    QETCGTMVRALMPCLPFVQGKEKEPSKGCCSGAKRLDGETKTGPQRVHACECIQTAMKTYSDIDGKLVS
NL12_PARJU    EETCGTVVRALMPCLPFVQGKEKEPSKGCCSGAKRLDGETKTGLQRVHACECIQTAMKTYSDIDGKLVS
NL13_PARJU    -ETCGTVVGALMPCLPFVQGKEKEPSKGCCSGAKRLDGETKTGPQRVHACECIQTAMKTYSDIDGKLVS
NL22_PARJU    EGPCGKVVHHIMPCLKFVKGEEKEPSKSCCSGTKKLSEEVKTTEQKREACKCIVAATKGISGIKNELVA
NL21_PARJU    -EACGKVVQDIMPCLHFVKGEEKEPSKECCSGTKKLSEEVKTTEQKREACKCIVRATKGISGIKNELVA

NLTP_PYRCO    GLPGKCGVN-VPY-KISTSTNCATVK----------------------------------------
NLTP_MALDO    ALPGKCGVN-VPY-KISTSTNCATVK----------------------------------------
NLT1_PRUDO    ALPGKCGVN-VPY-KISASTNCATVK----------------------------------------
NLT1_PRUAR    ALPGKCGVN-IPY-KISASTNCATVK----------------------------------------
NLT1_PRUPE    ALPGKCGVH-IPY-KISASTNCATVK----------------------------------------
NLTP_PRUAV    SIPGKCGVN-VPY-KISPSTNCATVK----------------------------------------
NLTP_MAIZE    SIPSKCGVS-IPY-TISTSTDCSRVN----------------------------------------
NLTA_WHEAT    SIPPKCGVN-LPY-TISLNIDCSRV-----------------------------------------
NLT1_HORVU    LLPSKCNVN-VPY-TISPDIDCSRIY----------------------------------------
NLT6_AMBAR    EVSTKCGVK-PDFPAVDKNLDCSKLPV---------------------------------------
NL11_PARJU    EVPKHCGIVDSKLPPIDVNMDCKTVGVVPRQPQLPVSLRHGPVTGPSDPAHKARLERPQIRVPPPAPEKA
NL12_PARJU    EVPKHCGIVDSKLPPIDVNMDCKTLGVVPRQPQLPVSLRHGPVTGPSDPAHKARLERPQIRVPPPAPEKA
NL13_PARJU    EVPKHCGIVDSKLPPIDVNMDCKTLGVLHYKGN---------------------------------
NL22_PARJU    EVPKKCGIT-TTLPPITADFDCSKIESTIFRGYY--------------------------------
NL21_PARJU    --PKKCDIK-TTLPPITADFDCSKIQSTIFRGYY--------------------------------
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**Fig. 4.1** Amino acid sequences of major nsLTP1 and nsLTP1-like allergens characterised from different plant species and organs (SWISS-PROT accession code).

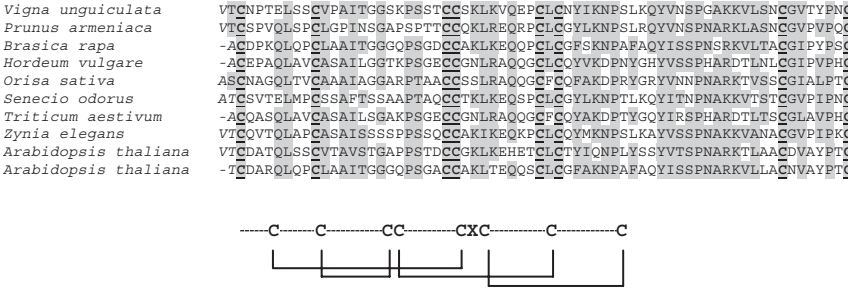


**Fig. 4.2** Amino acid sequences and cysteine pairing of nsLTP2 from different plant species. Only nsLTP2 from *Brassica rapa* has been recognised as a pollen allergen.

protein sequences registered in the data banks shows that other proteins share an nsLTP cysteine signature, but they display low sequence identity with recognised nsLTPs for the rest of their amino acid sequence. Consequently, it becomes important to limit *true nsLTPs* to proteins that have significant identity (above 30% when the cysteines are not taken into account) and a similar polypeptide chain length with no or few gaps being introduced when the alignment is performed either with nsLTP1 or with nsLTP2. For example, Ace-AMP1, an antimicrobial protein from onion seeds [7], has a nsLTP cysteine signature and similar polypeptide chain length, and no gaps are necessary to align with other nsLTP1s. However, with only about 25% identity with the closest nsLTP1 sequence (cysteines are excluded) and two tryptophan residues, Ace-AMP1 is considered to be an nsLTP1-like protein. Similarly, the allergens Par j1 and Par j2 from the pollen of *Parietaria judaica* should be considered as nsLTP1-like proteins (Fig. 4.1; Table 4.1).

Concerning relationships of food allergy with plant proteins, most of the nsLTP allergens identified until now belong to the nsLTP1 family [8–16] (Table 4.1). While nsLTP1s are recognised as allergens in plant-derived foods (fruits, cereals) and in pollen, nsLTP2 has been reported as a potent allergen only in the pollen of *Brassica rapa* [17](Table 4.1). The cysteine signature is also displayed by other small plant protein allergens with no sequence identity with nsLTP such as the hydrophobic protein from soybean (HPS), 2S [18] albumins [19] and cereal α-amylase inhibitors [20, see also Chapter 2 in this volume]. Interestingly, HPS with an nsLTP2 cysteine pairing has been described as the major allergen from soybean dust [21], while 2S albumins with an nsLTP1 cysteine pairing are considered as major food allergens [22, see Chapters 2 and 3]. Although, less closely related to nsLTPs and containing five disulphide bonds, it is worthy of note that the wheat 0.19 α-amylase inhibitor has the HPS cysteine pairing [20] and belongs to the cereal α-amylase inhibitor family identified as responsible for bakers' asthma [23–24, see Chapter 5]. Finally, nsLTP1 allergens can display less than 40% sequence identity (cysteines excluded, for example fruit vs barley) (Fig. 4.1). This explains the low or absence of cross-reaction of IgE between some nsLTP allergens [13, 25]. A large variation in sequence identity (27–81%) is also observed for wheat seed nsLTPs (M.-F. Gautier, personal communication). This means that, in organs such

**Table 4.1** Examples of identified nsLTP allergens in plants

| Plant | Organ/tissue | Allergen | LTP type | Accession |
|---|---|---|---|---|
| Common ragweed *Ambrosia artemisiifolia* | Pollen | Amb a6 | LTP1 | NLT6_AMBAR |
| Mugwort *Artemisia vulgaris* | Pollen | Art v 3 | LTP1 | |
| Pellitory-of-the-Wall *Parietaria judaica* | Pollen | Par j 1<br>Par j 2 | LTP1-like | NL21_PARJU<br>NL11_PARJU |
| Olive *Oleae europaea* | Pollen | Ole e7 | LTP1 | |
| Turnip *Brassica rapa* | Pollen | | LTP2 | BAA25680 |
| Chestnut *Castanea sativa* | Seed | | LTP1 | |
| Maize *Zea mays* | Seed | Zea m 14 | LTP1 | NLTP_MAIZE |
| Apple *Malus domestica* | Fruit | Mal d 3 | LTP1 | NLTP_MALDO |
| Apricot *Prunus armeniaca* | Fruit | Pru ar 3 | LTP1 | NLT1_PRUAR |
| Sweet cherry *Prunus avium* | Fruit | Pru av3 | LTP1 | NLTP_PRUAV |
| European plum *Prunus domestica* | Fruit | Pru d 3 | LTP1 | NLT1_PRUDO |
| Peach *Prunus persica* | Fruit | Pru p 3 | LTP1 | NLT1_PRUPE |
| Grape *Vitis vinifera* | Fruit | Vit v 1 | LTP1 | NLT4_VITSX |
| Pear *Pyrus communis* | Fruit | Pyr c3 | LTP1 | NLTP_PYRCO |

as seeds, different nsLTPs can be potent allergens. Therefore, the presence of an nsLTP cysteine signature, rather than the close sequence homologies with nsLTPs, can be taken as the first indication that a considered protein could be a novel plant allergen.

## 4.3 The nsLTP fold: a tool for identifying plant allergens?

The three-dimensional structures of different seed nsLTP1s (wheat, maize, rice, barley) have been determined by both NMR spectroscopy and X-ray crystallography. This folding is characterised by a four-helix bundle, surrounded in part by a *C*-terminus formed by turns that displays a saxophone-like shape (Fig. 4.3a). The folding is stabilised by four disulphide bonds. The presence of these disulphide bonds is essential to maintain the structure of the protein [26] and is probably responsible for the high thermal stability of nsLTP1 [27–28]. The most interesting feature of this fold is the presence of a large internal cavity. The surface of this cavity is covered by the side chains of hydrophobic residues of the amphipathic helices and the *C*-terminal region. The size of this cavity

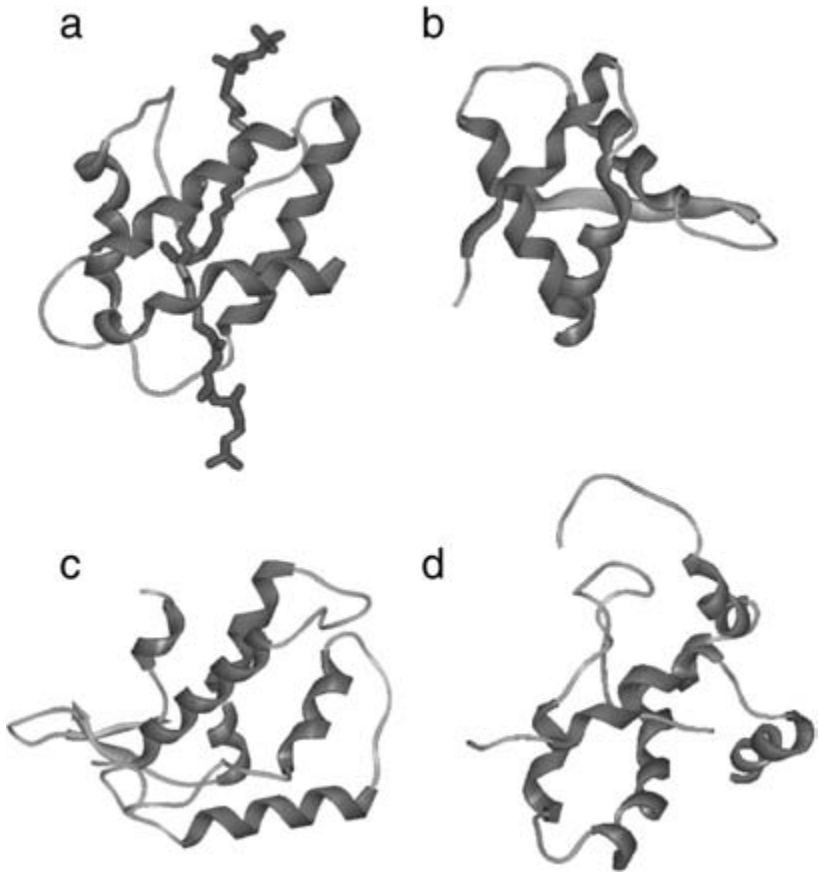


**Fig. 4.3** Three-dimensional structure of proteins that display an nsLTP1 fold. (a) wheat nsLTP1 complexed with two lysophosphatidylcholines (b) HPS (c) amylase inhibitor and (d) 2S albumin (napin). Disulphide bonds are not displayed.

is variable from one protein to another when comparing the structures of different lipid-free nsLTP1s [29–33]. In fact, this cavity is a tunnel following the long axis of the protein. Highly mobile water molecules are suspected to fill the tunnel since they are not visible in the crystals [30, 34]. It should be noted here that all the hydrophobic amino acids that are potent cleavage sites of pepsin are not accessible to the enzyme. This explains the high resistance of nsLTP1s to pepsin hydrolysis [25, 28]. Determination of the structures of different lipid–protein complexes have shown that the tunnel can adapt its volume to bind one or two monoacyl lipids [30, 33, 35–36] and a diacylated lipid [37]. This high plasticity of the tunnel is also highlighted by the fact that nsLTP1s are capable of binding different types of hydrophobic molecules including sphingolipids, prostaglandins, amphotericin B and other hydrophobic drugs [38–39]. For mono- and diacylated lipids, the complex is stabilised by a hydrogen bond between the tyrosine of the *C*-terminal region and the phosphate or carboxylate group of the lipid. When two lysophosphatidylcholines are bound to the wheat nsLTP1 [35],

the second lipid adopts a dissimilar orientation with its polar head protruding outside the protein, between helix H1 and H3. A similar orientation was observed in the case of the barley nsLTP1 complexed with either one molecule of palmitic coenzyme A or palmitic acid [40–41]. However, both tyrosine fluorescence and isothermal titration calorimetry experiments indicate that barley nsLTP1, like wheat nsLTP1, is capable of binding two monoacylated lipids [42]. Recent experiments suggest that the binding is a very complex dynamic process that leads, in solution, to proteins with one or two bound monoacylated lipids [Douliez, personal communication]. Irrespective of this, our experience from protein sequencing shows that nsLTP1s become relatively resistant to tryptic and chymotryptic cleavages, when lipids are bound in the tunnel [D. Marion, personal communication]. This can explain the resistance to other acidic fungal proteases [27]. Consequently, the nsLTP1 fold gives rise to proteins whose lipid-binding properties enhance their stability and resistance to proteolysis by the enzymes of the digestive tract. This is generally considered to be an important characteristic of small allergenic proteins [25, 43].

Structural information on nsLTP2 is still sparse. These proteins display a helical structure as revealed by CD spectroscopy and, as mentioned above, similar cysteine pairing except for the mismatch at the Cys-X-Cys motif [3]. If we assume similar folding properties for nsLTP1 and nsLTP2, the mismatch at the Cys-X-Cys motif can be explained by a rotation of the homologous H3 helix in nsLTP2. This phenomenon is probably related to the hydrophobicity of the X residue, which is always hydrophilic in nsLTP1 and hydrophobic in nsLTP2. As a first attempt, we have suggested that such a mismatch could be related to the absence or presence of an internal lipid-binding tunnel, an hypothesis that predicts different modes of binding for nsLTP1 and nsLTP2 [3]. However, the recent structure determination of a liganded wheat nsLTP2 by NMR spectroscopy shows that this nsLTP2 also has a cavity [44].

Other plant proteins that display the nsLTP cysteine signature also have an *LTP fold* such as the hydrophobic protein of soybean [18] and, to a lesser extent, 2S seed storage proteins [19] and cereal α-amylase inhibitors [20] (Fig. 4.3b,c,d; see also Chapter 2). These structural homologies between seed proteins have been already predicted from comparison of their cysteine patterns [45]. Of more interest is the fact that Ace-AMP1, an antimicrobial protein isolated from onion seeds [7], has a fold almost superimposable to that of nsLTP1 [46]. However, as mentioned above, except for the cysteine signature, the amino acid sequence of this antimicrobial protein markedly diverges from that of nsLTP1. It is worthy of note that AceAMP1 is not capable of transferring lipids, probably because the tunnel is filled up with tryptophan and phenylalanine side chains. The absence of a lipid-binding cavity is also observed for the other protein allergens with an nsLTP fold. This means that there is no relationship between the allergenicity and the lipid-binding properties of this structural family of protein allergens. Although no data are available on the stability and resistance to proteases of these plant proteins, it appears that (i) the nsLTP cysteine signature and close cysteine pairing are associated with an nsLTP or nsLTP-like fold, and (ii) the search for nsLTP folds among the α-helical protein families in the protein data banks (including those of other organisms) could be a means to detect putative protein allergens. Finally, nsLTPs are an interesting model

to study the relationship between the structure and the allergenicity of proteins, since most of the small protein allergens characterised until now are all β-sheet proteins [47].

### 4.4 The biological function of nsLTPs: possible relationships to allergy

Although numerous data are available on the genetics, structure and biosynthesis of nsLTPs, their real function is still a matter of discussion. All of the available structural, biochemical and physiological data confirm that nsLTPs are not involved in intracellular lipid trafficking, but do indicate a key role of nsLTPs in the resistance of plants towards biotic and abiotic stresses [1–2, 48–49], which is consistent with their expression in epidermal tissues of plants and their extracellular location [50–56]. To explain these biological roles, nsLTPs could be involved (i) in the formation of hydrophobic protective layers (cutin and suberin), and (ii) in the inhibition of fungal growth by interfering with the permeability of their membranes. Cutin, a hydrophobic polymer of most aerial plant organs, is a polyester of fatty acids, fatty alcohols and hydroxy-fatty acids, in which waxes are embedded. Suberin is a complex polymer composed of phenolic compounds, glycerol, dicarboxy-fatty acids and hydroxy-fatty acid derivatives [57]. Concerning the formation of protective hydrophobic layers, we have recently suggested on the basis of the spatial and temporal expression of the corresponding genes, that nsLTP1s are involved in the formation of cutin layers while nsLTP2s are involved in the formation of suberin layers [2]. A role of nsLTP1 in the formation of sporopollenin, a β-carotenoid, xanthophyll and fatty acid polymer, specific to the pollen coat has been also suggested [58]. The precise role of nsLTPs in the formation of cutin/suberin/sproropollenin layers is still unknown, but it can be suggested that they transport the hydrophobic monomers to the extracellular hydrophobic-hydrophilic cutin–cell wall interface where they polymerise and are generally located. From a structural standpoint, the plasticity of the hydrophobic tunnel is obviously an advantage for the formation of hydrophobic layers, since they are composed of a large variety of saturated and unsaturated fatty acid derivatives with hydroxy, carboxy and/or keto functions [57]. In contrast, the presence of a tunnel is not necessary for the antimicrobial activity of nsLTP1s, since wheat and maize nsLTP1s do not display antimicrobial activities while the onion LTP1-like protein, Ace-AMP1, does. Although we have no structural information on antifungal nsLTP1s, their high sequence identity with the rice and maize nsLTP1s suggest that they have also an internal lipid-binding tunnel. These antifungal nsLTP1s could therefore participate in both the formation of plant hydrophobic layers and the inhibition of fungal growth by modifying the permeability of the cell membranes. It should be noted that both the functions may contribute synergistically to the resistance of plants to fungal pathogens by protecting plant cells from invading fungi and by repairing wounded tissues. Concerning relationships between allergy and the defence functions of nsLTP1, it is interesting to note that other pathogenesis-related (PR) proteins are also major plant allergens [59, see also Chapters 6 and 8]. This relationship is clearly not direct but could be related to the structural stability of these proteins that have to work in an physicochemically hostile environment. Furthermore, as observed for nsLTPs, it also appears that they are generally

concentrated in the external part of organs (peel of fruits, seed coat, surface of pollen) and are immediately accessible to human cells of the digestive and the respiratory tracts.

Recently, it has been shown that nsLTP1s are recognised by a specific receptor located on the surface of plant cell membranes [60]. This receptor is also the receptor of elicitins [61–62], small proteins secreted by fungal pathogens of the genera *Phytophthora* and *Pythium* [63–64]. Elicitins are 10 kDa monomeric proteins with an α-helix fold stabilised by three disulphide bonds, which provides a hydrophobic cavity for binding lipids with a high binding specificity for sterols, although they are also capable of binding fatty acids [65–68]. These proteins do not share sequence identity with nsLTPs, do not display a nsLTP fold, and have a hydrophobic cavity rather than a tunnel. Elicitins are sterol transfer proteins, while nsLTPs cannot bind and transfer sterols. However, it is possible to superimpose some helical domains of nsLTP1 and elicitins, which is in agreement with their affinity and competition for the same membrane receptor. This binding to a common receptor is related to differences in the mode of binding and the biological responses that are triggered. Elicitins trigger a hypersensitive, systemic, acquired and non-specific resistance that is related, at the cell level, to a complex cascade of signalling pathways [69]. These biological and cellular responses can be inhibited by nsLTP1s [60].

It is interesting to note here that nsLTP allergens trigger a hypersensitive reaction in human cells that resembles, to some extent, the hypersensitive response of plant cells to fungal elicitor. Therefore, it is possible to speculate that a similar subtle recognition process could occur on animal membranes, i.e. the recognition of a specific receptor and a subsequent cell signalling response. Interestingly, another cell wall protein [70], soybean hydrophobic protein, has an nsLTP fold and allergenic properties [21], opening an interesting question about its possible interaction with the nsLTP1-elicitin receptor. Therefore, to go further with this speculative comparison, we can enquire whether a binding assay to the plant nsLTP/elicitin receptor would provide a predictive assay to screen protein allergens?

### 4.5 Food processing: a way to decrease the allergenicity of nsLTPs?

Most of the described allergies involving nsLTPs are either to pollen or fruits. NsLTP from cereals, especially barley and wheat, are rarely described as allergens. In the case of barley and brewing, it is interesting to note some allergic patients may be sensitive to beers while others are not [71]. All these observations are in agreement with an impact of food processing on the development of food allergy. In this regard, malting and brewing are good examples of how processing can decrease the allergenicity of plant-derived foods.

It has been shown that nsLTP1 and protein Z (a serpin-type proteinase inhibitor) constitute the major proteins of beer, where they contribute to the formation and stability of head foams [72]. Both of these proteins are allergens for patients with allergy to beer [73], but barley and malted barley contain many other proteins that dilute them. This means that some food processing systems can concentrate the most potent plant allergens. In contrast, wheat endosperm flours are generally used for baked products with most of the aleurone layer and embryo that have high concentrations

of nsLTPs [5–6] being eliminated by milling. Fortunately, it has been shown that most of the barley nsLTP1 is glycated through Maillard reactions during malting, especially when the water content of green malt decreases on heating [74–75]. Furthermore, the glycated protein is, in part, denatured during brewing due to both the reduction of disulphide bonds and the heating treatment on brewing [74–77]. Consequently, if brewing concentrates the most stable proteins, and therefore the most potent allergens, malting could lead to a decrease in their allergenicity by modification (glycation) of the nsLTP epitopic sites. Furthermore, partial reduction and denaturation on brewing should facilitate further hydrolysis by proteases from the digestive tract. This example suggests that decreasing heating treatment to avoid, for example, the loss of vitamins, could increase food allergy. In the case of wheat-baked products, the redox compounds that are generally added (ascorbic acid, cysteine, sulphite), as well as the disulphide bond rearrangements that are observed on heating and cooking wheat bread doughs, could decrease the allergen potential of nsLTP1 and related proteins.

### 4.6 Conclusion: what can we do to decrease allergy towards nsLTP allergen?

This brief survey of plant nsLTPs shows that these small proteins are interesting models to study the relationships between the structure and the biological and technological properties of a protein and its allergenic potential. Thus, the nsLTP helical fold characterised by high compactness and stability is shared by other plant allergens. Some of these allergenic proteins are also involved in host–pathogen interactions and signalling pathways that, to some extent, resemble the hypersensitive reaction involved in allergy. NsLTPs have interesting biological properties for improving plant resistance to microbial pathogens and functional properties for food end-uses so that a challenging conflict arises between technology, agronomic and human health interests. So, in this context, what is possible or not possible to do to minimise the impact of nsLTP allergens?:

1. Using plant biotechnology to increase resistance to microbial pathogens by overexpressing some nsLTPs [78] should be avoided or limited to expression in organs that are not used in food processing. In the same way, all treatments intended to improve plant resistance to virus and other microbial pathogens, through fungal elicitors (oligosaccharides, lipids, proteins) for example, that could induce synthesis of potent nsLTPs and other PR-protein allergens should only be used at stages of plant development that avoid the accumulation of these proteins in organs (e.g. seeds, fruits) used for food.

2. Although reduction in allergenicity by genetic engineering was demonstrated as feasible in rice seed [79] and grass pollen [80], the presence of multigene families and their key functions in the physiology of plants may make it difficult to reduce nsLTP content by using antisense and co-suppression of gene expression. Furthermore, the physiological and genetic drawbacks of such a strategy [see 81 for a review] are emphasised both by the variation in responses to an allergen and, at the present time, the non-acceptance of GMOs by consumers.

3. The stabilities of nsLTP proteins towards thermal denaturation and proteolytic hydrolysis seem to be the key parameters relating to their allergenicity. Consequently, all technological treatments that could decrease this stability and protease resistance should lead to a significant decrease in allergenicity. This could be achieved using redox agents (e.g. ascorbic acid, cysteine), or by improving the yield of the Maillard reaction during cooking. As observed for nsLTPs, such treatments do not necessarily lead to a loss of their functional properties but can result in an improvement, for example, in the case of beer. The removal of nsLTPs by ultrafiltration could also contribute to the production of hypoallergenic fruit beverages [27].

4. Finally, any progress in the production of hypoallergenic plant-derived foods or in immunotherapy will require determination of the epitopic sites recognised by IgE, from patients allergic to different nsLTPs and structurally-related proteins (e.g. soybean hydrophobic protein, amylase inhibitors, 2S albumins). For example, identification of the cross-reactive epitopes of the major apple and birch allergen allowed production of a mutant with low allergenicity [82] that could be a good candidate for immunotherapy [83].

## References


1. Kader, J.C. (1996) Lipid transfer proteins in plants. *Ann Rev Plant Physiol Plant Mol Biol* **47**, 627–654.
2. Douliez, J.P., Michon, T., Elmorjani, K. & Marion, D. (2000) Mini Review: structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J Cereal Sci* **32**, 1–20.
3. Douliez, J.P., Pato, C., Rabesona, H., Molle, D. & Marion, D. (2001) Disulfide bond assignment, lipid transfer activity and secondary structure of a 7 kDa plant lipid transfer protein, LTP2. *Eur J Biochem* **268**, 1400–1403.
4. Monnet, F.P., Dieryck, W., Boutrot, F., Joudrier, P. & Gautier, M.F. (2001) Purification, characterisation and cDNA cloning of a type 2 (7 kDa) lipid transfer protein from *Triticum durum*. *Plant Sci* **161**, 747–755.
5. Dubreil, L., Gaborit, T., Bouchet, B. *et al.* (1998) Spatial and temporal distribution of the major isoforms of puroindolines (puroindoline-a and puroindoline-b) and non specific lipid transfer protein (ns-LTPe1) of *Triticum aestivum* seeds. Relationships with their *in vitro* antifungal properties. *Plant Sci* **138**, 121–135.
6. Neumann, G.M., Condron, R., Thomas, I. & Polya, G.M. (1994) Purification and sequencing of a family of wheat lipid transfer protein homologues phosphorylated by plant calcium-dependent protein kinase. *Biochim Biophys Acta* **1209**, 183–190.
7. Cammue, B.P., Thevissen, K., Hendriks, M. *et al*. (1995) A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. *Plant Physiol* **109**, 445–455.
8. Sánchez-Monge, R., Lombardero, M., García-Sellés, F.J., Barber, D. & Salcedo, G. (1999) Lipid-transfer proteins are relevant allergens in fruit allergy. *J Allergy Clin Immunol* **103**, 514–519.
9. Pastorello, E.A., Farioli, L., Pravettoni, V. *et al*. (1999) The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *J Allergy Clin Immunol* **103**, 520–526.
10. Pastorello, E.A., Pravettoni, V., Farioli, L. *et al*. (1999) Clinical role of a lipid transfer protein that acts as a new apple-specific allergen. *J Allergy Clin Immunol* **104**, 1099–1106.
11. Pastorello, E.A., Ortolani, C., Baroglio, C. *et al*. (1999) Complete amino acid sequence determination of the major allergen of peach (*Prunus persica*) Pru p 1. *Biol Chem* **380**, 1315–1320.
12. Pastorello, E.A., D'Ambrosio, F.P., Pravettoni, V. *et al*. (2000) Evidence for a lipid transfer protein as the major allergen of apricot. *J Allergy Clin Immunol* **105**, 371–377.
13. Pastorello, E.A., Farioli, L., Pravettoni, V. *et al*. (2000) The maize major allergen, which is responsible for food-induced allergic reactions, is a lipid transfer protein. *J Allergy Clin Immunol* **106**, 744–751.
14. Conti, A., Fortunato, D., Ortolani, C. *et al*. (2001) Determination of the primary structure of two lipid transfer proteins from apricot (*Prunus armeniaca*). *J Chromatogr B* **756**, 123–129.
15. Pastorello, E.A., Farioli, L., Pravettoni, V. *et al*. (2001) Characterization of the major allergen of plum as a lipid transfer protein. *J Chromatogr B* **756**, 95–103.

16. Pastorello, E.A., Farioli, L., Robino, A.M., Trambaioli, C., Conti, A. & Pravettoni, V. (2001) A lipid transfer protein involved in occupational sensitization to spelt. *J Allergy Clin Immunol* **108**, 145–146.
17. Toriyama, K., Hanaoka, K., Okada, T. & Watanabe, M. (1998) Molecular cloning of a cDNA encoding a pollen extracellular protein as a potential source of a pollen allergen in *Brassica rapa*. *FEBS Lett* **424**, 234–238.
18. Baud, F., Pebay-Peyroula, E., Cohen-Addad, C., Odani, S. & Lehmann, M.S. (1993) Crystal structure of hydrophobic protein from soybean: a member of a new cysteine-rich family. *J Mol Biol* **231**, 877–887.
19. Rico, M., Bruix, M., Gonzales, C., Monsalve, R.I. & Rodriguez, R. (1996) 1H NMR Assignment and global fold of napin BnIb, a representative 2S albumin seed protein. *Biochemistry* **35**, 15672–15682.
20. Oda, Y., Matsunaga, T., Fukuyama, K., Miyazaki, T. & Morimoto, T. (1997) Tertiary and quaternary structures of 0.19 α-amylase inhibitor from wheat kernel determined by X-ray analysis at 2.06 Å resolution. *Biochemistry* **36**, 13503–13511.
21. Gonzalez, R., Varela, J., Carreira, J. & Polo, F. (1995) Soybean hydrophobic protein and soybean hull allergy. *Lancet* **346**, 48–49.
22. Pastorello, E.A., Pompei, C., Pravettoni, V. *et al.* (2001) Lipid transfer proteins and 2S albumins as allergens. *Allergy* **5**, 45–47.
23. Armentia, A., Sanchez-Monge, R., Gomez, L., Barber, D. & Salcedo, G. (1993) *In vivo* allergenic activities of eleven purified members of a major allergen family from wheat and barley flour. *Clin Exp Allergy* **23**, 410–415.
24. Amano, M., Ogawa, H. & Kojima, K. (1998) Identification of the major allergens in wheat flour responsible for baker's asthma. *Biochem J* **330**, 1229–1234.
25. Asero, R., Mistrello, G., Roncarolo, D. *et al.* (2001) Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* **122**, 20–32.
26. Désormeaux, A., Blochet, J.E., Pézolet, M. & Marion, D. (1992) Amino acid sequence of a non-specific wheat phospholipid binding protein and its conformation revealed by infrared and Raman spectroscopy, Role of disulphide bridges and phospholipids in stabilizing the α-helix. *Biochim Biophys Acta* **1121**, 137–152.
27. Brenna, O., Pompei, C., Ortolani, C., Pravettoni, V., Farioli, L. & Pastorello, E.A. (2000) Technological processes to decrease the allergenicity of peach juice and nectar. *J Agric Food Chem* **48**, 493–497.
28. Lindorff-Larsen, K. & Winther, J.R. (2001) Surprisingly high stability of barley lipid transfer protein, LTP1, towards denaturant, heat and proteases. *FEBS Lett* **488**, 145–148.
29. Gincel, E., Simorre, J.P., Caille, A., Marion, D., Ptak, M. & Vovelle, F. (1994) Three-dimensional structure in solution of a wheat lipid transfer protein from multidimensional 1H-NMR data – a new folding for lipid carriers. *Eur J Biochem* **226**, 413–422.
30. Shin, D.H., Lee, J.Y., Qwang, K.Y., Kim, K.K. & Suh, S.W. (1995) High resolution crystal structure of the non specific lipid transfer protein from maize seedlings. *Structure* **3**, 189–199.
31. Heinemann, B., Andersen, K., Nielsen, P., Bech, L. & Poulsen, F. (1996) Structure in solution of a four helix lipid binding protein. *Protein Sci* **5**, 13–23.
32. Lee, J.Y., Min, K., Cha, H., Shin, D.H., Hwang, K.Y. & Suh, S.W. (1998) Rice non-specific lipid transfer protein: the 1.6 Å crystal structure in the unliganded state reveals a small hydrophobic cavity. *J Mol Biol* **276**, 437–448.
33. Gomar, J., Petit, M.C., Sodano, P. *et al.* (1996) Solution structure and lipid binding of a non specific lipid transfer protein extracted from maize seeds. *Protein Sci* **5**, 565–577.
34. Gomar, J., Sodano, P., Sy, D. *et al.* (1998) Comparison of solution and crystal structures of maize nonspecific lipid transfer protein: a model for a potential in vivo lipid carrier protein. *Proteins* **31**, 160–171.
35. Charvolin, D., Douliez, J.P., Cohen-Addad, C., Marion, D. & Pebay-Peyroula, E. (1999) The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 Å resolution. *Eur J Biochem* **264**, 562–568.
36. Han, G.W., Lee, J.Y., Song, H.K. *et al.* (2001) Structural basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution X-ray crystallography. *J Mol Biol* **308**, 263–278.
37. Sodano, P., Caille, A., Sy, D., De Person, G., Vovelle, F., Marion, D. & Ptak, M. (1997) 1H NMR and fluorescence studies of complexation of DMPG by wheat lipid transfer protein, Global fold of the complex. *FEBS Lett* **416**, 130–134.
38. Tassin-Moindrot, S., Caille, A., Douliez, J.P., Marion, D. & Vovelle, F. (2000) The wide binding of a wheat nonspecific lipid transfer protein. Solution structure of a complex with prostaglandin B2. *Eur J Biochem* **267**, 1117–1124.
39. Pato, C., Le Borgne, M., Le Baut, G., Le Pape, P., Marion, D. & Douliez, J.P. (2001) Potential application of plant lipid transfer proteins for drug delivery. *Biochem Pharmacol* **62**, 555–560.

40. Lerche, M.H., Kragelund, B.B., Bech, L.M. & Poulsen, F.M. (1997) Barley lipid-transfer protein complexed with palmitoyl CoA: the structure reveals a hydrophobic binding site that can expand to fit both large and small lipid-like ligands. *Structure* **5**, 291–306.
41. Lerche, M.H. & Poulsen, F.M. (1998) Solution structure of barley lipid transfer protein complexed with palmitate. Two different binding modes of palmitate in the homologous maize and barley nonspecific lipid transfer proteins. *Protein Sci* **7**, 2490–2498.
42. Douliez, J.P., Jegou, S., Pato, C., Molle, D., Tran, V. & Marion, D. (2001) Binding of two monoacylated lipid monomers by the barley lipid transfer protein, LTP1, as viewed by fluorescence, isothermal titration calorimetry and molecular modelling. *Eur J Biochem* **268**, 384–388.
43. Astwood, J.D., Leach, J.N. & Fuch, R.L. (1996) Stability of food allergens to digestion in vitro. *Nat biotechnol* **14**, 1269–1273.
44. Pons, J.L., de Lamotte, F., Gautier, M.F. & Delsuc, M.A. (2003) Refined solution structure of a liganded type 2 wheat non-specific Lipid Transfer Protein. *J Biol Chem* **278**, 14249–14256.
45. Kreis, M., Forde, B.G., Rahman, S., Miflin, B.J. & Shewry, P.R. (1985) Molecular evolution of the seed storage proteins of barley, rye and wheat. *J Mol Biol* **183**, 499–502.
46. Tassin, S., Broekaert, W.F., Marion, D. *et al*. (1998) Solution structure of Ace-AMP1, a potent antimicrobial protein extracted from onion seeds. Structural analogies with plant nonspecific lipid transfer proteins. *Biochemistry* **37**, 3623–3637.
47. Bredehorst, R. & David, K. (2001) What establishes a protein as an allergen? *J Chromatogr B* **756**, 33–40.
48. Garcia-Olmedo, F., Molina, A., Segura, A. & Moreno, M. (1995) The defensive role of nonspecific lipid-transfer proteins in plants. *Trends Microbiol* **3**, 72–74.
49. Broekaert, W.F., Cammue, B.P.A., De Bolle, M.F.C., Thevissen, K., De Samblanx, G.W. & Osborn, R.W. (1997) Antimicrobial peptides from plants. *Cri Rev Plant Sci* **16**, 297–323.
50. Sossountzov, L., Ruiz-Avila, L., Vignols, F. *et al*. (1991) Spatial and temporal expression of a maize lipid transfer protein gene. *Plant Cell* **3**, 923–933.
51. Kotilainen, M., Helariutta, Y., Elomaa, P., Paulin, L. & Teeri, T.H. (1994) A corolla- and carpel-abundant, non-specific lipid transfer protein gene is expressed in the epidermis and parenchyma of *Gerbera hybrida* var. *Regina* (Compositae). *Plant Mol Biol* **26**, 971–978.
52. Hollenbach, B., Schreiber, L., Hartung, W. & Dietz, K.J. (1997) Cadmium leads to stimulated expression of the lipid transfer protein genes in barley: implications for the involvement of lipid transfer proteins in wax assembly. *Planta* **203**, 9–19.
53. Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A. & De Vries, S.C. (1991) Cell specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* **3**, 907–921.
54. Couthos-Thevenot, P., Jouenne, T., Maes, O. *et al*. (1993) Four 9 kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid transfer proteins. *Eur J Biochem* **217**, 885–889.
55. Thoma, S., Kaneto, Y. & Somerville, C.R. (1993) A nonspecific lipid transfer protein from *Arabidopsis* is a cell wall protein, *Plant J* **3**, 427–436.
56. Pyee, J., Yu, H. & Kolattukuddy, P.E. (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Arch Biochem Biophys* **311**, 460–468.
57. Kolattukudy, P.E. (2001) Polyesters in higher plants *Adv Biochem Engineer* **71**, 1–49.
58. Foster, G.D., Robinson, S.W., Blundell, R.P. *et al*. (1992) A *Brassica napus* mRNA encoding a protein homologous to phospholipid transfer proteins, is expressed specifically in the tapetum and developing microspores. *Plant Sci* **84**, 187–192.
59. Hoffmann-Sommergruber, K. (2000) Plant allergens and pathogenesis-related proteins. What do they have in common? *Int Arch Allergy Immunol* **122**, 155–166.
60. Buhot, N., Douliez, J.P., Jacquemard, A. *et al*. (2001) A lipid transfer protein binds to a receptor involved in the control of plant defence responses. *FEBS Lett* **509**, 27–30.
61. Wendehenne, D., Binet, M.N., Blein, J.P., Ricci, P. & Pugin, A. (1995) Evidence for specific, high-affinity binding sites for a proteinaceous elicitor in tobacco plasma membrane. *FEBS Lett* **374**, 203–207.
62. Osman, H., Vauthrin, S., Mikes, V. *et al*. (2001) Mediation of elicitin activity on tobacco is assumed by elicitin–sterol complexes. *Mol Biol Cell* **12**, 2825–2834.
63. Ricci, P., Bonnet, P., Huet, J.C. *et al*. (1989) Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco. *Eur J Biochem* **183**, 555–563.
64. Panabieres, F., Ponchet, M., Allasia, V., Cardin, L. & Ricci, P. (1997) Characterization of border species among *Pythiaceae*: several *Pythium* isolates produce elicitins, typical proteins from *Phytophthora* spp. *Mycol Res* **101**, 1459–1468.
65. Mikes, V., Milat, M.L., Ponchet, M., Panabieres, F., Ricci, P. & Blein, J.P. (1998) Elicitins excreted by *Phytophthora* are a new class of sterol carrier proteins. *Biochem Biophys Res Comm* **245**, 133–139.
66. Mikes, V., Milat, M.L., Ponchet, M., Ricci, P. & Blein, J.P. (1997) The fungal elicitor cryptogein is a sterol carrier protein. *FEBS Lett* **416**, 190–192.

67. Vauthrin, S., Mikes, V., Milat, M.L. *et al.* (1999) Elicitins trap and transfer sterols from micelles, liposomes and plant plasma membranes. *Biochim Biophys Acta* **1419**, 335–342.
68. Osman, H., Mikes, V., Milat, M.L. *et al.* (2001) Fatty acids bind to the fungal elicitor cryptogein and compete with sterols. *FEBS Lett* **489**, 55–58.
69. Ponchet, M., Panabieres, F., Milat, M.-L. *et al.* (1999) Are elicitins cryptograms in plant-fungi communication? A review of the plant and cell responses to elicitin treatments and analysis of signaling involved. *Cell Mol Life Sci* **56**, 1020–1047.
70. Gijzen, M., Miller, S.S., Kuflu, K., Buzzell, R.I. & Miki, B.L. (1999) Hydrophobic protein synthesized in the pod endocarp adheres to the seed surface. *Plant Physiol* **120**, 951–959.
71. Asero, R., Mistrello, G., Roncarolo, D., Amato, S. & van Ree, R. (2001) A case of allergy to beer showing cross-reactivity between lipid transfer proteins. *Ann Allergy Asthma Immunol* **87**, 65–67.
72. Sørensen, S.B., Bech, L.M., Muldbjerg, T.B. & Breddam, K. (1993) Barley lipid transfer protein 1 is involved in beer foam formation. *MBAA Technical Quaterly* **30**, 136–145.
73. Garcia-Casado, G., Crespo, J.F., Rodriguez, J. & Salcedo, G. (2001) Isolation and characterization of barley lipid transfer protein and protein Z as beer allergens. *J Allergy Clin Immunol* **108**, 647–649.
74. Jégou, S., Douliez, J.P., Molle, D., Boivin, P. & Marion, D. (2001) Evidence of the glycation and denaturation of LTP1 during the malting and brewing process. *J Agric Food Chem* **49**, 4942–4949.
75. Marion, D., Jégou, S., Douliez, J.P., Gaborit, T. & Boivin, P. (2001) Generation of foaming proteins along the malting and brewing processes. *Proc Eur Brew Conv* **28**, 631–638.
76. Bech, L.M., Vaag, P., Heinemann, B. & Breddam, K. (1995) Throughout the brewing process barley lipid transfer protein1 (LTP1) is transformed into a more foam-promoting form. *Proc Eur Brew Conv* **22**, 561–568.
77. Jégou, S., Douliez, J.P., Molle, D., Boivin, P. & Marion, D. (2000) Purification and structural characterization of LTP1 polypeptides from beer. *J Agric Food Chem* **48**, 5023–5029.
78. Molina, A. & Garcia-Omedo, F. (1997) Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *Plant J* **12**, 669–675.
79. Tada, Y., Nakase, M., Adachi, T. *et al.* (1996) Reduction of 14–16 kDa allergenic proteins in transgenic rice plants by antisense gene. *FEBS Lett* **391**, 341–345.
80. Bhalla, P.L., Swoboda, I. & Singh, M.B. (2001) Reduction of allergenicity of grass pollen by genetic engineering. *Int Arch Allergy Immunol* **124**, 51–54.
81. Shewry, P.R., Tatham, A.S. & Halford, N.G. (2001) Genetic modification and plant food allergens: risks and benefits. *J Chromatogr* **756**, 327–335.
82. Son, D.Y., Scheurer, S., Hoffmann, A., Haustein, D. & Vieths, S. (1999) Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d1, the major apple allergen, and Bet v1, the major pollen allergen. *Eur J Nutr* **34**, 201–215.
83. Swoboda, I., De Weerd, N., Bhalla, P.L. *et al.* (2002) Mutants of the major ryegrass pollen allergen, Lol p 5, with reduced IgE-binding capacity: candidates for grass pollen-specific immunotherapy. *Eur J Immunol* **32**, 270–280.

# 5 The Cereal α-Amylase/Trypsin Inhibitor Family Associated with Bakers' Asthma and Food Allergy

GABRIEL SALCEDO, ROSA SÁNCHEZ-MONGE,
GLORIA GARCÍA-CASADO, ALICIA ARMENTIA,
LUIS GOMEZ and DOMINGO BARBER

## 5.1 The α-amylase/trypsin inhibitor family in wheat, barley and rye flour

A link between plant defence proteins and plant food allergens has been established during the last decade [1, 2]. Constitutive and inducible proteinaceous inhibitors of heterologous hydrolytic enzymes are included in such plant defence proteins [3, 4]. A large proportion of the salt-soluble proteins from flour of wheat, barley and rye, members of the grass tribe *Triticeae*, belong to a single α-amylase/trypsin inhibitor family, which is associated with allergies provoked by inhalation or ingestion of cereal-derived products [2, 5, 6].

### 5.1.1 *General characteristics and types of inhibitors*

Members of this Triticeae inhibitor family are 12–16 kDa polypeptides, usually rich in glutamine plus asparagine and proline residues, and with four or five disulphide bridges essential for their inhibitory activity. Some of them, besides being salt-soluble proteins, are also extracted with chloroform/methanol mixtures, and have therefore been named CM proteins [5, 6].

The inhibitor subunits are synthesised by membrane-bound polysomes, as precursors comprising a signal peptide that precedes the *N*-terminal sequence of the mature protein [7, 8]. Their synthesis is apparently restricted to the seed storage tissue (endosperm) and peaks between 15 and 20 days after pollination, and their amount decreases drastically with the onset of germination [8, 9].

Sequence identity between members of the family ranges from around 30% to 95%. A weak relationship with a range of other proteins including the 2S storage proteins and sulphur-rich domains from cereal prolamins, has been proposed [10, 11] (see also Chapter 2 in this volume).

The Triticeae inhibitors are active towards heterologous α-amylases from insects, mites and mammals, or against trypsin-like proteases. In contrast, the endogenous hydrolytic enzymes present in the cereal seed are not affected [5, 6].

Bifunctional inhibitors of both α-amylase and trypsin have been identified in other Poaceae species, such as ragi (*Eleusine coracana*) [12, 13].

Three types of α-amylase inhibitors, monomeric, homodimeric and heterotetrameric, have been characterised in wheat and barley, based on their degree of aggregation [14–21; see Fig. 5.1]. The active forms of the tetrameric inhibitors include three different subunits, one of them in two copies. Each subunit by itself shows either no inhibitory activity or only residual activity [20, 21]. All identified trypsin inhibitors belong to the monomeric type [5].

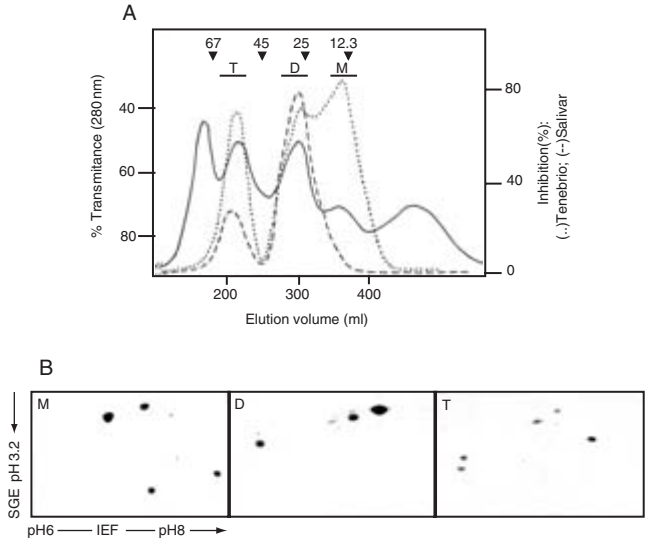


**Fig. 5.1** **A**. Types of α-amylase inhibitors differing in their degree of aggregation can be detected when a protein preparation enriched in inhibitors from bread wheat is fractionated by gel-filtration under non-dissociating conditions. **B**. Monomeric (**M**), dimeric (**D**) and tetrameric (**T**) inhibitor fractions show different and specific subunits in two-dimensional electrophoretic maps (IEF: isoelectrofocusing; SGE: starch-gel electrophoresis).

### 5.1.2 *Inhibitor subfamilies*

Up to 23 members of the inhibitor family have been isolated and characterised in wheat, barley and rye. Based on their degree of sequence identity (>70%) and the similarity in inhibitory and aggregative properties (although exceptions have been described; see below), the identified members can be grouped in ten different subfamilies (Fig. 5.2). Complete amino acid sequences have been obtained for most inhibitor subunits, either by direct protein sequencing [22–26] or deduced from the nucleotide sequences of the corresponding cDNA clones [17, 27–32] (Fig. 5.3). However, only *N*-terminal sequences are still available in some cases [18, 19, 33–35; see Fig. 5.2].

Close structural and functional relationships generally exist between subunits within the same subfamily. However, several examples of differential behaviour between closely related members within and between species have been described. Thus, different anti-trypsin activities have been found in genetic variants of the barley inhibitor BTI-CMe [36], while substantial changes in aggregative properties and specificity towards α-amylases are shown by two isoforms of the tetrameric subunit BTAI-CMa of the same species [37]. Similar differences have also been revealed when comparing a homodimeric rye inhibitor (RAI-3) with its wheat and barley counterparts (the tetrameric subunits WTAI-CM2 and BTAI-CMa; see Fig. 5.2) [33]. Finally, differences in post-translational modifications, affecting the attachment of complex asparagine-linked glycans, have been detected in homologous inhibitors of barley and rye (BMAI-1 versus RAI-1, respectively) [33].

| Inhibitor subunit | Activity towards | Aggregation | *N*-terminal amino acid sequence |
|---|---|---|---|
| WMAI-1 (syn 0.28) | α-Amylase | M | SGPWSWCNPATGYKVSALTGCRAMVKLQ-CVGSQVPEAVL |
| WMAI-2* | | M | M D M R P |
| BMAI-1 (syn Hor v1) | α-Amylase | M | S-PGEWCWPGMGYPVYPFPRCRALVKSQ-CAGGQVVESIQ |
| RAI-1* | | D | H M * |
| WDAI-1 (syn 0.53) | α-Amylase | D | SGPWM-CYPGQAFQVPALPGCRPLLKLQ-CNGSQVPEAVL |
| WDAI-2 (syn 0.19) | | D | A R |
| WDAI-3* | | D | Y K V L |
| RDAI-1* | | D | N V* |
| BDAI-1 | α-Amylase | D | SGPWMWCDPEMGHKVSPLTRCRALVKLE-CVGNRVPEDVL |
| BDP* | Unknown | D | ERDYGEYCRVGKSIPINPLP* |
| Sec c 1* | | D | QC S SN V ACREYV* |
| WTAI-CM1 | α-Amylase (1st subunit) | T | TGPYCYAGMGLPINPLEGCREYVASQTCGIS-ISGSAVS |
| WTAI-CM2 | | T | P S Q VGIV P |
| WTAI-CMa | | T | Q S Q VT-A P |
| RAI-3* | | D | TK Q * |
| WTAI-CM16 | α-Amylase (2nd subunit) | T | IGNEDCTPWMSTLIYPLPSCRDYVEQQACRIET-PGS--- |
| WTAI-CM17 | | T | T N E M PPYL |
| BTAI-CMb | | T | V S TA P PPYL |
| WTAI-CM3 | α-Amylase (3rd subunit; two copies) | T | ---SGSCYPGVAFRTNLLPHCRDYVLQQTCTF-T-PGSKL |
| BTAI-CMd | | T | AAAATD S P G AVL |
| BTI-CMe | Trypsin | M | F-GDSCAPGDALPHNPLRACRTYVVSQICHQGPRLLTSD- |
| RTI | | M | SV GQ V L M G V |
| BTI-CMc | Trypsin | M | TSIYTCYEGMGLPVNPLQGCRFYVASQTCGAVPLLPIEV- |

**Fig. 5.2** Amino acid sequences of subfamilies of inhibitor subunits characterised in wheat (W), barley (B) and rye (R). Only differences in amino acid sequences within each subfamily are shown.* = only *N*-terminal sequence has been obtained. The asterisk also indicates the end of the available sequence. M = monomeric; D = homodimeric; T = heterotetrameric; A = α-amylase; T = trypsin; I = inhibitor.

### 5.1.3 *Inhibitory activities*

Activity against α-amylases from insects, mites, mammals and/or bacteria has been reported for this inhibitor family [6, 38, 39]. However, the three inhibitor types, monomeric, homodimeric and tetrameric, display high variation, both in specificity and effectiveness, towards enzymes from different origins (Fig. 5.4). Furthermore, distinctive inhibitory patterns are also found between members of two subfamilies within the same inhibitor type, or, even more, between highly related active forms (such as the wheat and barley tetramers).

The *in vitro* effects of the inhibitors against the amylase activity of coleopteran and lepidopteran pests, mainly predators of stored cereal seed, should be emphasised in relation to their involvement in resistance mechanisms [40–42]. On the other hand, the inhibition of the α-amylase from *Dermatophagoides pteronyssinus* (a house-dust mite), which is one of the allergens (Der p 4) described in this species, suggests that interactions between mite and cereal allergens (α-amylase/inhibitor complexes) might occur in house-dust mite-infected flours [38].

The inhibitory sites of these inhibitors have not been fully determined, but have been investigated using different approaches. The inhibitors have been proposed to

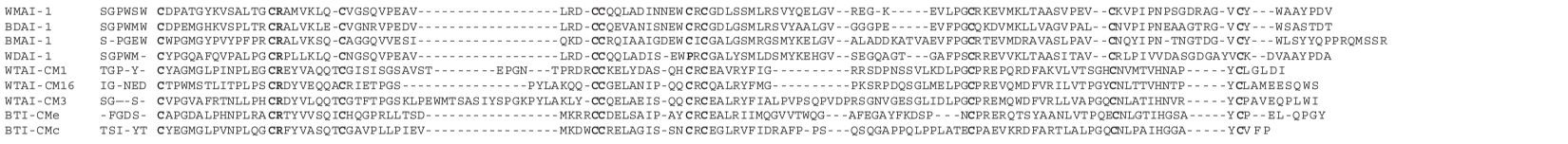


**Fig. 5.3** Comparison of the complete amino acid sequences of representative members of each inhibitor subfamily included in Fig. 5.2.

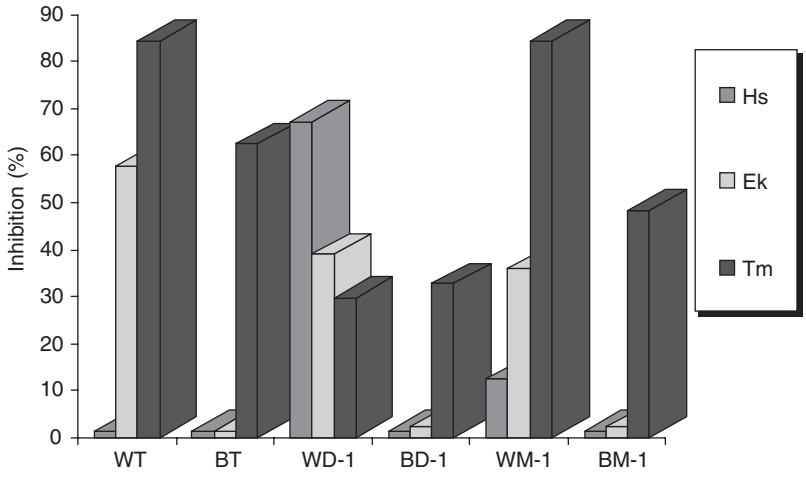


**Fig. 5.4** Inhibition patterns against α-amylases from different origins differ among inhibitor types, subfamilies and active forms (i.e. tetramers) from wheat and barley. Tm = *Tenebrio molitor*: Insect, *Coleoptera*; Ek = *Ephestia kuehniella*: Insect, *Lepidoptera*; Hs = Human saliva.

bind to the catalytic site of the α-amylase competitively, forming 1:1 complexes by a two-step model mechanism [39–43]. Site-directed mutagenesis of a wheat monomeric inhibitor (WMAI-1) identified the *N*-terminal region and position 58 as essential for its activity [44]. Determination of the three-dimensional structure of the bifunctional α-amylase/trypsin inhibitor from ragi seeds (RBI), and its complex with the α-amylase from the coleopteran pest *Tenebrio molitor*, provides evidence of the mechanism of inhibition [13, 43]. RBI consist of a globular four-helix motif with a simple *up-and-down* topology. A short fragment forms an antiparallel β-sheet between α-helices 3 and 4. Two regions in RBI, the *N*-terminal fragment Ser 1–Ala 11 and the segment including residues Pro 52–Cys 55, have been identified as interacting with the substrate-binding groove to directly target the active site of the insect α-amylase. WDAI-2 (synonymous 0.19) is the only α-amylase inhibitor of the Triticeae whose tertiary and quaternary structures have been determinated [45]. Each subunit exhibits a topology quite similar to that of BTI, with five α-helices and two short antiparallel β-strands, suggesting that members of the inhibitor family have a common fold. The interface between subunits in the active dimeric form seems to include mainly α-helix 4, the *C*-terminal loop and the turn connecting α-helices 1 and 2 (where the reactive site of the trypsin inhibitors is located; see below). Interestingly, most residues that make up the interface are hydrophobic.

Among the trypsin inhibitors of the Triticeae family, that from barley, named BTI-CMe, is the best characterised. Homologous proteins have been identified in maize (MTI) and ragi (RBI) as well as rye (RTI; see Fig. 5.2) [12, 13, 46]. The barley inhibitor affects both bovine trypsin and the trypsin-like activity of some lepidopteran pests [25, 42]. It is also active against the Hageman factor (factor XII-a) of the blood-clotting cascade and against kallikrein [47]. No anti-chymotrypsin, -papain or -pepsin activity has been detected for BTI-CMe [48]. In contrast with the bifunctional nature

of the maize and ragi homologues, no significant inhibition of insect α-amylases has been found for any BTI-CMe genetic variants analysed so far [36].

The trypsin reactive site of all these inhibitors is the motif proline–arginine–leucine, located around position 34 of their primary structures [5]. This motif is present in the trypsin-binding loop, where the conformation of the residues surrounding the scissile bond (arginine–leucine) allows strong binding of the inhibitor to trypsin in a substrate-like manner [13, 49].

The use of cereal α-amylase/trypsin inhibitor genes for obtaining transgenic insect resistance has been recently reviewed [50].

### 5.1.4 *Inhibitor genes: location and evolution*

The chromosomal location of genes encoding the different inhibitor subunits has been determined in bread wheat (*Triticum aestivum*; allohexaploid; genomes AABBDD), barley (*Hordeum vulgare*; diploid; genome HH) and rye (*Secale cereale*; diploid; genome RR) [18, 19, 27, 34, 35, 51–54]. The genomic organisation of the corresponding gene family is summarised in Table 5.1.

The data available to date indicate that the multigene family encoding inhibitor subunits is dispersed over five out of the seven homoeologous chromosome groups. It has been generated both by inter-chromosomal translocations and intra-chromosomal duplications, and most of the dispersion seems to have originated in an ancestral diploid species (the ancient progenitor of A, B, D, H and R genomes).

The differential expression of inhibitor types and subfamilies in wheat, barley and rye (see Fig. 5.2) suggests divergence in both the products encoded by the multigene family, and the regulatory mechanisms controlling subunit expression in each species. Two additional events have been shown in the alloploid species *T. aestivum*: silencing of the genes located in the A genome [28; see Table 5.1] and permanent heterosis involving positive intergenomic interactions in the case of heterotetrameric α-amylase inhibitors [21].

Some of the intra-chromosomal duplications encode subunits with very similar properties (WDAI-1 and -3 in wheat), whereas others have diverged, coding either for two different tetrameric inhibitor subunits (WTAI-CM16 and -CM3B) or for polypeptides differing more widely in their aggregative and inhibitory behaviour (BTAI-CMa and BTI-CMc).

Finally, very low intraspecific variability has been found for the different members of the family in wheat and barley, in contrast with the considerable variability detected within a given genome [3, 52]. This genetic profile has allowed the use of inhibitor subunits as biochemical markers in several phylogenetic studies [55, 56].

## 5.2 The inhibitor family and bakers' asthma

### 5.2.1 *Diversity of allergens associated with bakers' asthma*

Bakers' asthma is a type I, IgE-mediated allergic response to the inhalation of cereal flours. It is among the most frequent occupational respiratory disorders, and represents

**Table 5.1** A dispersed multigene family encodes the α-amylase/trypsin inhibitor subunits

| Subunits grouped in subfamilies | Chromosomal location of the encoding gene |
|---|---|
| WMAI-1 | 6DS |
| WMAI-2 | 6BS |
| BMAI-1 | 2H |
| RAI-1 | ? |
| WDAI-1 | 3BS |
| WDAI-2 | 3DS |
| WDAI-3 | 3BS |
| RDAI-1 | 3RS |
| BDAI-1 | 6H |
| BDP | ? |
| Sec c 1 | 4RL |
| WTAI-CM1 | 7DS |
| WTAI-CM2 | 7BS |
| BTAI-CMa | 7HS |
| RAI-3 | 4RL (7RS) |
| WTAI-CM16 | 4BS |
| WTAI-CM17 | 4DS |
| BTAI-CMb | 4HS |
| WTAI-CM3B | 4BS |
| WTAI-CM3D | 4DS? |
| BTAI-CMd | 4HL |
| BTI-CMe | 3HS |
| RTI | 3R |
| BTI-CMc | 7HS |

B,D = genomes of hexaploid wheat (*Triticum aestivum*; genomes AABBDD); H = barley genome (*Hordeum vulgare*; genome HH); R = rye genome (*Secale cereale*; genome RR); L,S = long and short chromosome arms, respectively. Other abbreviations as in Fig. 5.2.

an important clinical problem requiring compensation in European countries [57, 58]. Bakers, millers, pastry factory workers and farmers are the main occupational groups affected. The prevalence of asthma in these risk groups is around 15–30%.

The major cause of the bakers' asthma condition is the inhalation of wheat, barley and rye flour proteins [57–61]. IgE antibodies to a large number of these cereal proteins have been detected in the allergic subjects' sera. Salt-soluble proteins (albumins plus globulins) show the strongest IgE reactivities, at least in wheat [62, 63], but prolamins (typical storage proteins, not extractable by salt solutions) have also been implicated as potential allergens [63, 64].

In addition to cereal proteins, a wide array of other allergenic components have been associated with bakers' asthma. The most important ones include several additives used to enhance wheat flour quality for baking, such as *Aspergillus*-derived enzymes (mainly α-amylase) [60, 65, 66] and soybean flour proteins [67]. In addition, cereal flour contaminants, including mites, pests, moulds and pollens, have also been recorded [59, 61].

### 5.2.2 *α-Amylase inhibitors as major allergens*

It has been mentioned above that the most relevant protein fraction from wheat flour for bakers' asthma is the water/salt soluble fraction comprising albumins and globulins. Up to 70 different IgE-binding spots have been identified in two-dimensional gel electrophoresis maps of this fraction [68], and wide variation in the reactive spots has been found among individual sera from patients suffering bakers' asthma [69]. In spite of this variation, most studies have shown that 12–16 kDa components, corresponding to members of the α-amylase/trypsin inhibitor family, are major salt-soluble allergens in wheat, barley and rye flours [68, 70–73]. In addition, fractions enriched in salt-soluble 14 kDa bands from wheat were shown to induce histamine release *in vitro* from peripheral basophiles obtained from wheat-flour sensitive subjects [74], while an uncharacterised 14 kDa allergen from rye increased CD23 expression in monocytes from atopic bakers [72].

Homology among inhibitor subunits of wheat, barley and rye (see Fig. 5.2) seems to partially account for the cross-reactivities between flours of these three cereals [73, 75, 76]. Furthermore, common IgE epitopes located in the sulphur-rich non-repetitive regions of prolamins and in the homologous sequences of inhibitor subunits [11] appear to explain the cross-reactions between wheat prolamins (mainly α-gliadins and glutenins) and salt-soluble proteins [64]. In particular, IgE epitopes shared by the homodimeric inhibitor WDAI-2 (synonymous 0.19) and α-gliadin are clearly indicated by RAST inhibition assays [64].

Our group has extensively demonstrated the *in vitro* IgE-binding capacity of purified inhibitor subunits, almost all of which belong to different subfamilies, using sera from Spanish patients [27, 35, 71, 76, 77–79]. Nonetheless, very different reactivities have been reported among these allergens, the glycosylated forms showing stronger responses (see below) [78, 79]. Members of the inhibitor family have also been identified as major allergens associated with wheat-induced asthma in patients from other countries. Thus, Fränken *et al.* [80] have identified a mixture of WDAI-1 (synonymous 0.53) and WDAI-2 using sera from German subjects while Amano *et al.* [81] have demonstrated the effects of WMAI-1 and WDAI-2 and a wheat homologue of barley BTI-CMe on Japanese individuals. The latter authors also showed stimulation of sulphidoleukotrienes by peripheral blood leucocytes from patients with the two former allergens, and also demonstrated that similar *in vitro* reactivity was exhibited by recombinant WDAI-2 (expressed in *E. coli*) and its natural form isolated from flour.

Only one approach has been used to locate IgE-binding epitopes in the primary structures of the inhibitor subunits [82]. Synthetic hexapeptides spanning the complete amino acid sequence of WMAI-1 allowed residues 9 to 26 to be identified as a high IgE-binding region.

### 5.2.3 *A relevant role of complex N-linked glycans*

Wide variation in *in vitro* reactivity has been shown among isolated inhibitor subunits (Fig. 5.5). The highest responses are observed with three glycosylated components, namely BMAI-1, WTAI-CM16* and BTAI-CMb* [27, 78]. BMAI-1 is a barley monomeric inhibitor of insect α-amylase, which has no known wheat equivalent and has

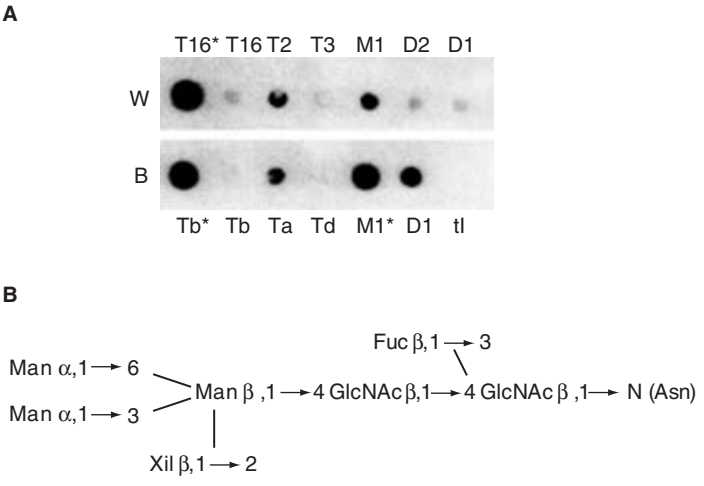


**Fig. 5.5** **A**. Different *in vitro* reactivities are shown by wheat (W) and barley (B) inhibitor subunits of heterotetrameric (T), homodimeric (D) and monomeric (M; tI: Trypsin) inhibitor types. Glycosylated proteins are indicated by an asterisk. **B**. Putative structure of the complex asparagine-linked glycan of inhibitor subunits BMAI-1, WTAI-CM16* and BTAI-CMb*. Man = mannose; Xil = xylose; GlcNAC = *N*-acetylglucosamine; Fuc = fucose.

not been found in a non-glycosylated form [27]. Wheat WTAI-CM16* and its barley counterpart BTAI-CMb* are glycosylated forms of the homologous tetrameric α-amylase inhibitor subunits WTAI-CM16 and BTAI-CMb, respectively [78]. Both glycosylated variants are about ten-fold less abundant than their non-glycosylated forms in the corresponding flour. On the other hand, glycosylation does not seem to affect the inhibitory properties of the subunits [83].

The recognition pattern of rabbit monospecific polyclonal antibodies to different types of complex asparagine-linked plant glycans has led to the proposed structure shown in Fig. 5.5B for the oligosaccharide attached to the single *N*-glycosylation site of the three modified subunits (BMAI-1, WTAI-CM16* and BTAI-CMb*) [79].

Several lines of evidence indicate an essential role of the glycan moieties in the IgE-binding capacity of the subunits. Firstly, their *in vitro* and *in vivo* (see below) reactivities are substantially stronger than those of their natural non-glycosylated counterparts [78]. Secondly, chemical or enzymatic deglycosylation resulted in total loss of their IgE-binding capacity [79]. Thirdly, only the endo-Lys peptide of BMAI-1 that harbours the single glycan is recognised by specific IgE from allergic patients [79].

The proposed *N*-glycan structure presented in Fig. 5.5B contains a β 1→2 xylosyl and an α 1→3 fucosyl residue. One or both of these are found in most plant and insect glycoproteins harbouring complex glycans, but not in mammalian glycoproteins [84]. To determine the importance of these carbohydrate residues in IgE binding, extracts from plant, insect, mite and mammalian origin, as well as purified glycoproteins (unrelated to the inhibitor family) with known glycan moiety structures have been analysed using sera from patients with bakers' asthma and with anti-plant complex glycan antibodies [79]. Based on this and other studies [85], it can be concluded that the presence of a β 1→2 xylosyl residue constitutes a key IgE-reactive determinant.

An important, although seemingly less critical role, can also be attributed to the α 1→3 fucose. The presence of these complex glycan residues probably explains to a large extent the cross-reactivities between plant glycoproteins, and between these glycoproteins and those from insects, such as bee venom phospholipase $A_2$.

### 5.2.4 *Differential* in vivo *responses of isolated inhibitor subunits*

Skin prick test has been used to ascertain the *in vivo* reactivity of up to 16 purified inhibitor subunits. Assays were carried out in two different groups of cereal-induced asthmatic patients, mainly sensitised to wheat [75] or rye-flour [76]. Responses to each protein tested are summarised in Table 5.2.

A high reactivity (67–87% of positive responses) has been shown by protein preparations enriched in components of the inhibitor family in both asthmatic groups. Moreover, about 95% of the subjects studied reacted to at least one isolated subunit from wheat, barley or rye. However, very different *in vivo* activities were displayed by the inhibitor proteins so far assayed (from around 10% to 75% of positive tests). In addition, wide differences are also found between homologous subunits belonging to the same subfamily but present in different species (as clearly shown in the rye group; see Table 5.2).

In agreement with the *in vitro* IgE-binding capacities discussed above, the three glycosylated subunits, BMAI-1, WTAI-CM16* and BTAI-CMb*, were found to be the strongest allergens among wheat-induced asthmatic patients, as indicated by skin sensitivity in prick tests. Interestingly, several non-glycosylated rye inhibitors (namely RDAI-1, Sec c 1 and RAI-3) seem to be major allergens in subjects sensitised to this cereal flour (positive responses in more than 50% of patients) in addition to BMAI-1.

### 5.2.5 *Inhibitors and occupational sensitisation in the wood industry*

The cereal α-amylase inhibitors are not only involved in classical bakers' asthma, the occupational disease prevailing among professionals who work in the flour industry (i.e. bakers and millers), but also in allergies associated with apparently unrelated activities, such as some wood industries [86, 87]. Cereal flour, particularly from rye, is used to increase the viscosity of the urea–formaldehyde glue employed to produce veneer panels, and two independent groups of wood-derivative factory workers have been shown to be sensitised to cereal proteins, mainly α-amylase inhibitor subunits [86, 87]. Specific IgE immunodetection and skin prick tests have demonstrated reactivity to the rye proteins Sec c 1, RAI-1 and RAI-3, as well as to other purified subunits from wheat and barley.

### 5.2.6 *Other Triticeae allergens potentially associated with bakers' asthma*

In addition to the α-amylase/trypsin inhibitor family, several other wheat or barley salt-soluble proteins have been shown to be related to occupational cereal flour-induced asthma. Most of them are enzymes, including amylases [88], peroxidase [89], acyl-CoA oxidase and fructose-bis-phosphate-aldolase [90], and glyceraldehyde-3-phosphate dehydrogenase and triosephosphate isomerase [69]. Wheat serpins, another putative

**Table 5.2** Isolated α-amylase inhibitor subunits induce *in vivo* responses as determined by skin prick test. Two groups of bakers' asthma patients sensitised mainly to wheat or rye have been tested

| Sample | Positive responses (Number of patients/%) | |
|---|---|---|
| | Wheat (N = 31) | Rye (N = 21) |
| WIP | 27 (87) | 14 (67) |
| BIP | 27 (87) | 15 (71) |
| RIP | – | 17 (81) |
| WMAI-1 | 9 (29) | – |
| BMAI-1 | 15 (48) | 16 (76) |
| RAI-1 | – | 6 (29) |
| WDAI-1 | 5 (16) | 1 (5) |
| RDAI-1 | – | 18 (86) |
| BDAI-1 | 9 (29) | – |
| BDP | – | 6 (33) |
| Sec c 1 | – | 15 (71) |
| WTAI-CM2 | 11 (35) | 3 (14) |
| RAI-3 | – | 12 (57) |
| WTAI-CM16 | 7 (22) | – |
| WTAI-CM16* | 14 (45) | – |
| BTAI-CMb | 9 (29) | – |
| BTAI-CMb* | 14 (45) | – |
| WTAI-CM3 | 11 (35) | – |
| BTI-CMe | 8 (26) | – |

Inhibitor subunits are grouped in subfamilies. * = glycosylated subunits.
W = wheat; B = barley; R = rye; IP = protein preparation enriched in inhibitors;
– = not determined. Other abbreviations as in Fig. 5.2.

class of protease inhibitors, have also been detected as IgE-binding components [69]. Interestingly, a barley serpin homologue, protein Z, has recently been identified as a beer allergen [91]. Further *in vivo* and *in vitro* studies are needed to assess the relevance of all these proteins in bakers' asthma. In fact, no skin prick tests on asthmatic patients have been performed with isolated allergens belonging to these novel families, except in the case of wheat peroxidase (around 60% of positive reactions) [89].

The major water-insoluble wheat proteins (prolamins), mainly α-gliadins, fast ω-gliadins and glutenins, also appear to be implicated in cereal hypersensitivity [64].

## 5.3 α-Amylase inhibitors and cereal-induced allergy by ingestion

### 5.3.1 *A second route of sensitisation*

Cereals, particularly wheat, rice and maize, are the staple foods consumed by the majority of the world population. Wheat is among the six major foods that account for food allergy in US children [92]. Most studies on hypersensitivity reactions following wheat ingestion focus on cereal-dependant, exercise-induced anaphylaxis, and on

atopic dermatitis [93–95]. Prolamins have been found to be the main IgE-binding components in both cases, and a peptide derived from the proline/glutamine-rich domains of these storage proteins appears to be an important IgE epitope [96].

In contrast, information on patients suffering from gastrointestinal symptoms on ingestion of foods containing wheat-derived products is very limited. Members of the inhibitor family have been identified in two significant reports [97, 98], thus indicating their capacity to sensitise susceptible atopic subjects by inhalation (bakers' asthma), and also by ingestion. James *et al.* [97] have identified WDAI-1 as a prominent allergen, binding IgE from six of the seven US patients tested. Simonato *et al.* [98] have shown reaction of uncharacterised 16 kDa inhibitor subunits in 10 out of 11 sera from wheat CAP-positive Italian patients. However, these authors have claimed that bread baking seems to result in a drastic decrease in IgE-binding to the 16 kDa allergen [99]. Finally, it should be mentioned that the wheat tetrameric α-amylase inhibitor subunit WTAI-CM3, but not its associated subunits WTAI-CM2 and -CM16, reacts with IgE from sera from Japanese subjects with wheat-related atopic dermatitis [100].

### 5.3.2 *The rice allergen family*

A family of inhibitors of heterologous α-amylases, homologous to those characterised in Triticeae species, are present in rice seeds [101–103]. It includes salt-soluble proteins of 14–16 kDa and isoelectric points between 6 and 8 which are active against human salivary α-amylase, but not against trypsin (although studies on their inhibitory properties are scarce). Up to ten different cDNA clones, grouped into four subfamilies, have been identified for rice inhibitors, indicating that these proteins are encoded by a multigene family [101, 104, 105]. However, most of the corresponding polypeptides have not been isolated.

A relationship between the rice, wheat and barley allergens (inhibitors) is supported by similarities in their structures and expression patterns. RA17 (also named RAP or 16 kDa allergen), the best characterised rice member of the family, shows 21–53% amino acid sequence identity with wheat and barley inhibitor subunits (Fig. 5.6) [5], with the ten cysteine residues and intramolecular disulphide bridges being conserved [106]. Furthermore, the tissue (endosperm-specific) and temporal (peaking at 10 to 20 days after flowering) expression patterns of rice inhibitors mirror those found for wheat and barley homologues [102].

Although the allergenicity of the rice inhibitor family is well established [103, 107, 108], additional studies (i.e. *in vivo* assays) are still needed. Data on differences in reactivity

```
RA17    DHHQVYSPGEQCRPGISYPTYSLPQCRTLVRRQCVGRGASAADEQVW
BMAI-1  ------SPGEWCWPGMGYPVYPFPRCRALVKSQCAG-GQVV--ESIQ

RA17    QDCCRQLAAVDDGWCRCGALDHMLSGIYRELGATEAGHP--MAEVFP
BMAI-1  KDCCRQIAAIGDEWCICGALGSMRGSMYKELGVALADDKATVAEVFP

RA17    GCRRGDLERAAASLPAFCNVDIPN--GPGGVCYWLGYPRTPR--TGH
BMAI-1  GCRTEVMDRAVASLPAVCNQYIPNTNGTDGVCYWLSYYQPPRQMSSR
```

**Fig. 5.6** Alignment of amino acid sequences of the major rice (RA17) and barley (BMAI-1) allergens.

between members are also limited [103]. The main approach to elucidate the allergenic properties of the rice proteins focused on the RA17 16 kDa allergen, using sera (n = 36) from Japanese patients with atopic dermatitis, with or without bronchial asthma [108]. The high responses in IgE immunoblotting analysis (recognised by 69% of the sera tested), positive RAST assays (all sera) and the histamine-release test from human leukocytes have proved that RA17 is a major allergen in rice grain. Furthermore, this conclusion is supported by significant positive correlations between the isolated allergen and a crude rice seed protein extract in RAST and histamine-release assays. IgE cross-reactive epitopes between RA17 and unidentified wheat and maize components have been revealed by RAST-inhibition experiments.

Finally, it should be mentioned that attempts to reduce the 14–16 kDa allergenic proteins in rice seeds have been made, using the antisense gene strategy [109]. Transgenic plants expressing the RA17 antisense RNA have been obtained, and lower amounts (around 25% comparing with parental wild-type rice) of 14–16 kDa proteins and their transcripts have been detected in their seed. Whether or not this putative hypoallergenic rice is useful for allergic patients remains to be determined.

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## References

1. Salcedo, G., Díaz-Perales, A. & Sánchez-Monge, R. (1999) Fruit allergy: plant defence proteins as novel potential panallergens. *Clin Exp Allergy* **29**, 1158–1160.
2. Breiteneder, H. & Ebner, C. (2000) Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* **106**, 27–36.
3. García-Olmedo, F., Salcedo, G., Sánchez-Monge, R., Gomez, L., Royo, J. & Carbonero, P. (1987) Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxford Surv Plant Mol Cell Biol* **4**, 275–334.
4. Shewry, P.R. & Lucas, J.A. (1997) Plant proteins that confer resistance to pest and pathogens. *Adv Bot Res* **26**, 135–192.
5. Carbonero, P., Salcedo, G., Sánchez-Monge, R. *et al*. (1993) A multigene family from cereals which encodes inhibitors of trypsin and heterologous α-amylases. In: *Innovations of Proteases and their Inhibitors*, Aviles, F.X. (ed.). Berlin, Walter de Gruyter, pp. 333–348.
6. Carbonero, P. & García-Olmedo, F. (1999) A multigene family of trypsin/α-amylase inhibitors from cereals. In: *Seed Proteins*, Shewry, P.R. & Casey, R. (eds). The Netherlands, Kluwer Academic Publishers, pp. 617–633.
7. García-Olmedo, F., Salcedo, G., Sánchez-Monge, R. *et al*. (1992) Trypsin/α-amylase inhibitors and thionins: possible defence proteins from barley. In: *Barley: Genetic, Biochemistry, Molecular Biology and Biotechnology*, Shewry, P.R. (ed.). Wallingford, CAB International, pp. 335–350.
8. Paz-Ares, J., Ponz, F., Aragoncillo, A. *et al*. (1983) *In vivo* and *in vitro* synthesis of CM-proteins (A-hordeins) from barley (*Hordeum vulgare* L.). *Planta* **157**, 74–80.
9. Pace, W., Parlamenti, R., Rab, A., Silano, V. & Vittozzi, L. (1978) Protein α-amylase inhibitors from wheat flour. *Cereal Chem* **55**, 244–254.
10. Shewry, P.R., Lafiandra, D., Salcedo, G. *et al*. (1984) *N*-terminal amino acid sequences of chloroform/methanol-soluble proteins and albumins from endosperms of wheat, barley and related species. *FEBS Lett* **175**, 359–363.

11. Kreis, M., Forde, B.G., Rahman, S., Miflin, B.J. & Shewry, P.R. (1985) Molecular evolution of the seed storage proteins of barley, rye and wheat. *J Mol Biol* **183**, 499–502.
12. Shivaraj, B. & Pattabiraman, T.N. (1981) Natural plant enzyme inhibitors. Characterization of an unusual α-amylase/trypsin inhibitor from ragi (*Eleusine coracana* Geartn). *Biochem J* **193**, 29–36.
13. Strobl, S., Mülhahn, P., Berstein, R. *et al*. (1995) Determination of the three-dimensional structure of the bifunctional α-amylase/trypsin inhibitor from ragi seeds by NMR spectroscopy. *Biochemistry* **34**, 8281–8293.
14. Buonocore, V., Petrucci, T. & Silano, V. (1977) Wheat protein inhibitors of α-amylase. *Phytochemistry* **16**, 811–820.
15. Buonocore, V. & Silano, V. (1986) Biochemical, nutritional and toxicological aspects of α-amylase inhibitors from plant foods. In: *Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods*, Friedman, M. (ed.). New York, Plenum Publishing Corporation, pp. 483–506.
16. Barber, D., Sánchez-Monge, R., Méndez, E., Lázaro, A., García-Olmedo, F. & Salcedo, G. (1986) New α-amylase and trypsin inhibitors among the CM-proteins of barley (*Hordeum vulgare*). *Biochim Biophys Acta* **869**, 115–118.
17. Lázaro, A., Sánchez-Monge, R., Salcedo, G., Paz-Ares, J., Carbonero, P. & García-Olmedo, F. (1988) A dimeric inhibitor of insect α-amylase from barley. Cloning of the cDNA and identification of the protein. *Eur J Biochem* **172**, 129–134.
18. Sánchez-Monge, R., Gomez, L., García-Olmedo, F. & Salcedo, G. (1989) New dimeric inhibitor of heterologous α-amylases encoded by a duplicated gene in the short arm of chromosome 3B of wheat (*Triticum aestivum* L.). *Eur J Biochem* **183**, 37–40.
19. Gomez, L., Sánchez-Monge, R., Lopez-Otín, C. & Salcedo, G. (1991) Wheat inhibitors of heterologous α-amylases. Characterization of major components from the monomeric class. *Plant Physiol* **96**, 768–774.
20. Sánchez-Monge, R., Gomez, L., García-Olmedo, F. & Salcedo, G. (1986) A tetrameric inhibitor of insect α-amylases from barley. *FEBS Lett* **207**, 105–109.
21. Gómez, L., Sánchez-Monge, R., García-Olmedo, F. & Salcedo, G. (1989) Wheat tetrameric inhibitors of insect α-amylases: alloploid heterosis at the molecular level. *Proc Natl Acad Sci USA* **86**, 3242–3246.
22. Kashlan, N. & Richardson, M. (1981) The complete amino acid sequence of a major wheat protein inhibitor of α-amylase. *Phytochemistry* **20**, 1781–1784.
23. Maeda, K., Hase, T. & Matsubara, H. (1983) Complete amino acid sequence of an α-amylase inhibitor in wheat kernel. *Biochim Biophys Acta* **743**, 52–57.
24. Maeda, K., Kakabayashi, S. & Matsubara, H. (1985) Complete amino acid sequence of an α-amylase inhibitor in wheat kernel (0.19-inhibitor). *Biochim Biophys Acta* **828**, 213–221.
25. Odani, S., Koide, T. & Ono, T. (1983) The complete amino acid sequence of barley trypsin inhibitor. *J Biol Chem* **258**, 7998–8003.
26. Lyons, A., Richardson, M., Tatham, S. & Shewry, P.R. (1987) Characterization of homologous inhibitors of trypsin and α-amylase from seeds of rye (*Secale cereale*). *Biochim Biophys Acta* **916**, 305–310.
27. Mena, M., Sánchez-Monge, R., Gomez, L., Salcedo, G. & Carbonero, P. (1992) A major barley allergen associated with baker's asthma disease is a glycosylated monomeric inhibitor of insect α-amylase: cDNA cloning and chromosomal location of the gene. *Plant Mol Biol* **20**, 451–458.
28. García-Maroto, F., Maraña, C., Mena, M., García-Olmedo, F. & Carbonero, P. (1990) Cloning of cDNA and chromosomal location of genes encoding the three types of subunits of the wheat tetrameric inhibitor of insect α-amylase. *Plant Mol Biol* **14**, 845–853.
29. Medina, J., Hueros, G. & Carbonero, P. (1993) Cloning of cDNA, expression and chromosomal location of genes encoding the three types of subunits of the barley tetrameric inhibitor of insect α-amylase. *Plant Mol Biol* **23**, 535–542.
30. Gautier, M.F., Alary, R., Lullien, V. & Joudrier, P. (1991) Nucleotide sequence of a cDNA encoding the wheat (*Triticum durum* Desf) CM2 protein. *Plant Mol Biol* **16**, 333–334.
31. Lullien, V., Alary, R., Guirao, A., Joudrier, P. & Gautier, M.F. (1991) Isolation and nucleotide sequence of a cDNA encoding the bread wheat (*Triticum aestivum* L.) CM17 protein. *Plant Mol Biol* **17**, 1081–1082.
32. Gaddour, K. (1998) *Tesis Doctoral*. Madrid, Universidad Politécnica.
33. García-Casado, G., Sánchez-Monge, R., Lopez-Otín, C. & Salcedo, G. (1994) Rye inhibitors of animal α-amylases show different specificities, aggregative properties and IgE-binding capacities than their homologous proteins from wheat and barley. *Eur J Biochem* **224**, 525–531.
34. García-Casado, G., Sánchez-Monge, R., Lopez-Otín, C. & Salcedo, G. (1994) Rye chromosome arm 3RS encodes an homodimeric inhibitor of insect α-amylase. *Theor Appl Genet* **89**, 60–63.
35. García-Casado, G., Armentia, A., Sánchez-Monge, R., Sánchez, L.M., Lopez-Otín, C. & Salcedo, G. (1995) A major baker's asthma allergen from rye flour is considerably more active than its barley counterpart. *FEBS Lett* **364**, 36–40.

36. Moralejo, M.A., García-Casado, G., Sánchez-Monge, R. *et al*. (1993) Genetic variants of the trypsin inhibitor from barley endosperm show different inhibitory activities. *Plant Sci* **89**, 23–29.
37. García-Casado, G., Sánchez-Monge, R., Puente, X.S. & Salcedo, G. (1996) Divergence in properties of two closely related α-amylase inhibitors of barley. *Physiol Plant* **98**, 523–528.
38. Sánchez-Monge, R., García-Casado, G., Barber, D. & Salcedo, G. (1996) Interaction of allergens from house-dust mite and cereal flours: *Dermatophagoides pteronyssinus* α-amylase (Der p 4) and wheat and rye α-amylase inhibitors. *Allergy* **51**, 176–180.
39. Takase, K. (1994) Site-directed mutagenesis reveals critical importance of the catalytic site in the binding of α-amylase by wheat proteinaceous inhibitor. *Biochemistry* **33**, 7925–7930.
40. Gutierrez, C., Sánchez-Monge, R., Gomez, L., Ruiz-Tapiador, M., Castañera, P. & Salcedo, G. (1990) α-Amylase activities of agricultural insect pest are specifically affected by different inhibitor preparations from wheat and barley endosperm. *Plant Sci* **72**, 37–44.
41. Gutierrez, C., García-Casado, G., Sánchez-Monge, R., Gomez, L., Castañera, P. & Salcedo, G. (1993) The three inhibitor types from wheat endosperm are differentially active against α-amylases of Lepidoptera pest. *Entomol Exp Appl* **66**, 47–52.
42. Alfonso, J., Ortego, F., Sánchez-Monge, R. *et al*. (1997) Wheat and barley inhibitors active towards α-amylase and trypsin-like activities from *Spodoptera frugiperda* (Lepidoptera:Noctuidae). *J Chem Ecol* **23**, 1729–1741.
43. Strobl, S., Maskos, K., Wiegand, G., Huber, R., Gomis-Rüth, F.X. & Glockshuber, R. (1998) A novel strategy for inhibition of α-amylases: yellow meal worm α-amylase in complex with the ragi bifunctional inhibitor at 2.5 Å resolution. *Structure* **6**, 911–921.
44. García-Maroto, F., Carbonero, P. & García-Olmedo, F. (1991) Site-directed mutagenesis and expression in *Escherichia coli* of WMAI-1, a wheat monomeric inhibitor of insect α-amylase. *Plant Mol Biol* **17**, 1005–1011.
45. Oda, Y., Matsunaga, T., Fukuyama, K., Miyazaki, T. & Morimoto, T. (1997) Tertiary and quaternary structures of 0.19 α-amylase inhibitor from wheat kernel determined by X-ray analysis at 2.06 Å resolution. *Biochemistry* **36**, 13503–13511.
46. Mahoney, W.C., Hemodson, M.A., Jones, B., Powers, D.D., Corfman, R.S. & Reeck, G.R. (1984) Amino acid sequence and secondary structural analysis of the corn inhibitor of trypsin and activated Hageman factor. *J Biol Chem* **259**, 8412–8416.
47. Chong, G.L. & Reeck, G.R. (1987) Interaction of trypsin, β-factor XII a and plasma kallikrein with a trypsin inhibitor isolated from barley seeds: a comparison with the corn inhibitor of activated Hageman factor. *Thrombosis Res* **48**, 211–221.
48. Mikola, J. & Soulinna, E.M. (1969) Purification and properties of a trypsin inhibitor from barley. *Eur J Biochem* **9**, 555–560.
49. Behuke, C.A., Yee, V.C., Le Trong, I. *et al*. (1998) Structural determinants of the bifunctional corn Hageman factor inhibitor: X-ray crystal structure at 1.95 Å resolution. *Biochemistry* **37**, 15277–15288.
50. Carbonero, P., Díaz, I., Vicente-Carbajosa, J., Alfonso-Rubí, J., Gaddour, K. & Lara, P. (1999) Cereal α-amylase/trypsin inhibitors and transgenic insect resistance. In: *Genetics and Breeding for Crop Quality and Resistance*, Scarascia Mugnozza, G.T., Porceddu, E. & Pagnotta, M.A. (eds). The Netherlands, Kluwer Academic Publishers, pp. 147–158.
51. Hejgaard, J., Bjorn, S.E. & Nielsen, G. (1984) Rye chromosomes carrying structural genes for the major grain protease inhibitors. *Hereditas* **101**, 257–259.
52. Salcedo, G., Fra-Mon, P., Molina-Cano, J.L., Aragoncillo, C. & García-Olmedo, F. (1984) Genetics of CM-proteins (A-hordeins) in barley. *Theor Appl Genet* **68**, 53–59.
53. Fra-Mon, P., Salcedo, G., Aragoncillo, C. & García-Olmedo, F. (1984) Chromosomal assignment of genes controlling salt-soluble proteins (albumins and globulins) in wheat and related species. *Theor Appl Genet* **69**, 167–172.
54. Sánchez-Monge, R., Barber, D., Mendez, E., García-Olmedo, F. & Salcedo, G. (1986) Genes coding α-amylase inhibitors are located in the short arms of chromosomes 3B, 3D and 6D in wheat (*Triticum aestivum* L.). *Theor Appl Genet* **72**, 108–113.
55. García-Olmedo, F., Salcedo, G., Aragoncillo, C., Sánchez-Monge, R., Collada, C. & Gomez, L. (1988) Two-dimensional electrophoresis as a tool for structural and genetic studies of seed proteins from *Poaceae* and *Fagaceae*. *Electrophoresis* **9**, 719–727.
56. Moralejo, M., Romagosa, I., Salcedo, G., Sánchez-Monge, R. & Molina-Cano, J.L. (1994) On the origin of Spanish two-rowed barley. *Theor Appl Genet* **87**, 829–836.
57. Sandiford, C.P. (1996) Cereal-induced occupational asthma. *Clin Exp Allergy* **26**, 128–131.
58. Baur, X., Degens, P.O. & Sander, I. (1998) Baker's asthma: still among the most frequent occupational respiratory disorders. *J Allergy Clin Immunol* **102**, 984–997.
59. Savolainen, V. (1997) Baker's asthma: diversity of allergens. *Clin Exp Allergy* **27**, 1111–1113.

60. Baur, X. & Posch, A. (1998) Characterized allergens causing bakers' asthma. *Allergy* **53**, 562–566.
61. Alvarez, M.J., Tabar, A.I., Quirce, S. *et al*. (1996) Diversity of allergens causing occupational asthma among cereal workers as demostrated by exposure procedures. *Clin Exp Allergy* **26**, 147–153.
62. Baldo, B.A. & Wrigley, C.W. (1978) IgE antibodies to wheat flour components. *Clin Exp Allergy* **8**, 109–124.
63. Weiss, W., Vogelmeier, C. & Görg, A. (1993) Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma. *Electrophoresis* **14**, 805–816.
64. Sandiford, C.P., Tatham, A.S., Fido, R. *et al*. (1997) Identification of the major water/salt insoluble wheat proteins involved in cereal hypersensitivity. *Clin Exp Allergy* **27**, 1120–1129.
65. Quirce, S., Cuevas, M., Diez-Gomez, M.L. *et al*. (1992) Respiratory allergy to *Aspergillus*-derived enzymes in bakers' asthma. *J Allergy Clin Immunol* **90**, 970–978.
66. Baur, X., Chen, Z. & Sander, I. (1994) Isolation and denomination of an important allergen in baking additives: α-amylase from *Aspergillus oryzae* (Asp o II). *Clin Exp Allergy* **24**, 465–470.
67. Quirce, S., Polo, F., Figueredo, E., Gonzalez, R. & Sastre, J. (2000) Occupational asthma caused by soybean flour in bakers-differences with soybean-induced epidemic asthma. *Clin Exp Allergy* **30**, 839–846.
68. Posch, A., Weiss, W., Wheeler, C., Dunn, M.J. & Görg, A. (1995) Sequence analysis of wheat grain allergens separated by two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **16**, 1115–1119.
69. Sander, I., Flagge, A., Merget, R., Halder, T.M., Meyer, H.E. & Baur, X. (2001) Identification of wheat flour allergens by means of 2-dimensional immunoblotting. *J Allergy Clin Immunol* **107**, 907–913.
70. Pfeil, T., Schwabl, U., Ulmer, W.T. & Köning, W. (1990) Western blot analysis of water-soluble wheat flour (*Triticum vulgaris*) allergens. *Int Arch Allergy Appl Immunol* **91**, 224–231.
71. Gomez, L., Martin, E., Hernandez, D. *et al*. (1990) Members of the α-amylase inhibitors family from wheat endosperm are major allergens associated with baker's asthma. *FEBS Lett* **261**, 85–88.
72. Fränken, J., Stephan, U., Neuber, K., Bujanowski-Weber, J., Ulmer, W.T. & Köning, W. (1991) Characterization of allergenic components of rye and wheat flour (*Secale*, *Triticum vulgaris*) by western blot with sera of bakers: their effects on CD23 expression. *Int Arch Allergy Immunol* **96**, 76–83.
73. Sandiford, C.P., Tee, R.D. & Newman-Taylor, A.J. (1995) Identification of crossreacting wheat, rye, barley and soya flour allergens using sera from individuals with wheat-induced asthma. *Clin Exp Allergy* **25**, 340–349.
74. Theobald, K., Thiel, H., Kallweit, C., Ulmer, W. & Köning, W. (1986) Detection of proteins in wheat flour extracts that bind human IgG, IgE, and mouse monoclonal antibodies. *J Allergy Clin Immunol* **78**, 470–477.
75. Armentia, A., Sánchez-Monge, R., Gomez, L., Barber, D. & Salcedo, G. (1993) *In vivo* allergenic activities of eleven purified members of a major allergen family from wheat and barley flour. *Clin Exp Allergy* **23**, 410–415.
76. García-Casado, G., Armentia, A., Sánchez-Monge, R., Malpica, J.M. & Salcedo, G. (1996) Rye flour allergens associated with baker's asthma. Correlation between *in vivo* and *in vitro* activities and comparison with their wheat and barley homologues. *Clin Exp Allergy* **26**, 428–435.
77. Barber, D., Sánchez-Monge, R., Gómez, L. *et al*. (1989) A barley flour inhibitor of insect α-amylase is a major allergen associated with baker's asthma disease. *FEBS Lett* **248**, 119–122.
78. Sánchez-Monge, R., Gómez, L., Barber, D., Lopez-Otín, C., Armentia, A. & Salcedo, G. (1992) Wheat and barley allergens associated with baker's asthma. Glycosylated subunits of the α-amylase-inhibitor family have enhanced IgE-binding capacity. *Biochem J* **281**, 401–405.
79. García-Casado, G., Sánchez-Monge, R., Chrispeels, M.J., Armentia, A., Salcedo, G. & Gómez, L. (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology* **6**, 471–477.
80. Fränken, J., Stephan, U., Meyer, H.E. & Köning, W. (1994) Identification of alpha-amylase inhibitor as a major allergen of wheat flour. *Int Arch Allergy Immunol* **104**, 171–174.
81. Amano, M., Ogawa, H., Kojima, K. *et al*. (1998) Identification of the major allergens in wheat flour responsible for baker's asthma. *Biochem J* **330**, 1229–1234.
82. Walsh, B.J. & Howden, M.E.H. (1989) A method for the detection of IgE binding sequences of allergens based on a modification of epitope mapping. *J Immunol Methods* **121**, 275–280.
83. Moralejo, M.A., García-Casado, G., Sánchez-Monge, R. *et al*. (1993) Barley tetrameric inhibitor of insect α-amylase. Characterization of an allelic variant of the BTAI-CMb subunit. *J Cereal Sci* **17**, 107–113.
84. Faye, L., Johnson, K.D., Sturn, A. & Chrispeels, M.J. (1989) Structure, biosynthesis, and function of asparagine-linked glycans on plant glycoproteins. *Physiol Plant* **75**, 309–314.
85. Van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J. *et al*. (2000) β (1,2)-xylose and α (1,3)-fucose residues have a strong contribution in IgE binding of plant glycoallergens. *J Biol Chem* **275**, 11451–11458.

86. Armentia, A., García-Casado, G., Vega, J., Sánchez-Monge, R., Mendez, J. & Salcedo, G. (1997) Occupational allergy to rye flour in carpenters. *Allergy* **52**, 1151–1152.
87. Lopez-Rico, R., Moneo, I., Rico, A., Curiel, G., Sánchez-Monge, R. & Salcedo, G. (1998) Cereal α-amylase inhibitors cause occupational sensitization in the wood industry. *Clin Exp Allergy* **28**, 1286–1291.
88. Sandiford, C.P., Tee, R.D. & Newman Taylor, A.J. (1994) The role of cereal and fungal amylases in cereal flour hypersensitivity. *Clin Exp Allergy* **24**, 549–557.
89. Sánchez-Monge, R., García-Casado, G., Lopez-Otín, C., Armentia, A. & Salcedo, G. (1997) Wheat flour peroxidase is a prominent allergen associated with baker's asthma. *Clin Exp Allergy* **27**, 1130–1137.
90. Weiss, W., Huber, G., Engel, K.H. *et al*. (1997) Identification and characterization of wheat grain albumin/globulin allergens. *Electrophoresis* **18**, 826–833.
91. García-Casado, G., Crespo, J.F., Rodriguez, J. & Salcedo, G. (2001) Isolation and characterization of barley lipid transfer protein and protein Z as beer allergens. *J Allergy Clin Immunol* **108**, 647–649.
92. James, J.M. & Sampson, H.A. (1992) An overview of food hypersensitivity. *Pediatr Allergy Immunol* **3**, 67–68.
93. Palosuo, K., Alenius, H., Varjonen, E. *et al*. (1999) A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J Allergy Clin Immunol* **103**, 912–917.
94. Palosuo, K., Alenius, H., Varjonen, E., Kalkkinen, N. & Reunala, T. (2001) Rye γ-70 and γ-35 secalins and barley γ-3 hordein cross-react with ω-5 gliadin, a major allergen in wheat-dependent, exercise-induced anaphylaxis. *Clin Exp Allergy* **31**, 466–473.
95. Varjonen, E., Vainio, E. & Kalimo, K. (2000) Antigliadin IgE-indicator of wheat allergy in atopic dermatitis. *Allergy* **55**, 386–391.
96. Tanabe, S., Arai, S., Yanagihara, Y., Mita, H., Takahashi, K. & Watanabe, M. (1996) A major wheat allergen has a Glu-Glu-Glu-Pro-Pro motif identified as an IgE-binding epitope. *Biochem Biophys Res Comm* **219**, 290–293.
97. James, J.M., Sixbey, J.P., Helm, R.M., Bannon, G.A. & Burks, A.W. (1997) Wheat α-amylase inhibitor: a second route of allergic sensitization. *J Allergy Clin Immunol* **99**, 239–244.
98. Simonato, B., De Lazzari, F., Pasini, G. *et al*. (2001) IgE binding to soluble and insoluble wheat flour proteins in atopic and non-atopic patients suffering from gastrointestinal symptons after wheat ingestion. *Clin Exp Allergy* **31**, 1771–1778.
99. Simonato, B., Pasini, G., Giannattasio, M., Peruffo, A.D.B., De Lazzari, F. & Curioni, A. (2001) Food allergy to wheat products: the effect of bread baking and in vitro digestion on wheat allergenic proteins. A study with bread dough, crumb and crust. *J Agric Food Chem* **49**, 5668–5673.
100. Kusaba-Nakayama, M., Ki, M., Iwamoto, M., Shibata, R., Sato, M. & Imaizumi, K. (2000) CM3, one of the wheat α-amylase inhibitor subunits, and binding of IgE in sera from Japanese with atopic dermatitis related to wheat. *Food Chem Toxicol* **28**, 179–185.
101. Nakamura, R. & Matsuda, T. (1996) Rice allergenic protein and molecular-genetic approach for hypoallergenic rice. *Biosci Biotechnol Biochem* **60**, 1215–1221.
102. Adachi, T., Izumi, H., Yamada, T. *et al*. (1993) Gene structure and expression of rice seed allergenic proteins belonging to the α-amylase/trypsin inhibitor family. *Plant Mol Biol* **21**, 239–248.
103. Nakase, M., Adachi, T., Urisu, A. *et al*. (1996) Rice (*Oryza sativa* L.) α-amylase inhibitors of 14–16 kDa are potential allergens and product of a multigene family. *J Agric Food Chem* **44**, 2624–2628.
104. Izumi, H., Adachi, T., Fujii, N. *et al*. (1992) Nucleotide sequence of a cDNA clone encoding a major allergenic protein in rice seeds. Homology of the deduced amino acid sequence with members of α-amylase/trypsin inhibitor family. *FEBS Lett* **302**, 213–216.
105. Alvarez, A.M., Adachi, T., Nakase, M., Aoki, N., Nakamura, R. & Matsuda, T. (1995) Classification of rice allergenic protein cDNAs belonging to the α-amylase/trypsin inhibitor gene family. *Biochim Biophys Acta* **1251**, 201–204.
106. Izumi, H., Sugiyama, M., Matsuda, T. & Nakamura, R. (1999) Structural characterization of the 16 kDa allergen, RA17, in rice seeds. Prediction of the secondary structure and identification of intramolecular disulfide bridges. *Biosci Biotechnol Biochem* **63**, 2059–2063.
107. Matsuda, T., Sugiyama, M., Nakamura, R. & Torii, S. (1988) Purification and properties of an allergenic protein in rice grain. *Agric Biol Chem* **52**, 1465–1470.
108. Urisu, A., Yamada, K., Masuda, S. *et al*. (1991) 16-kilodalton rice protein is one of the major allergens in rice grain extract and responsible for cross-allergenicity between cereal grains in the *Poaceae* family. *Int Arch Allergy Appl Immunol* **96**, 244–252.
109. Toda, Y., Nakase, M., Adachi, T. *et al*. (1996) Reduction of 14–16 kDa allergenic proteins in transgenic rice plants by antisense gene. *FEBS Lett* **391**, 341–345.

# 6 Latex Allergy and Plant Chitinases

ROSA SÁNCHEZ-MONGE, ARACELI DÍAZ-PERALES,
CARLOS BLANCO and GABRIEL SALCEDO

## 6.1 The latex–fruit syndrome

The main connection between latex and plant food allergy seems to be the latex hevein and the hevein domain of plant class I chitinases. Therefore, this chapter deals with the involvement of these enzymes in the latex–fruit syndrome.

### 6.1.1 *Latex allergy and latex allergens*

Latex allergy refers to the IgE-mediated hypersensitivity caused by natural rubber latex (NRL), which is the milky sap produced by the laticiferous cells of the tropical rubber tree *Hevea brasiliensis*. NRL is a complex mixture of polyisoprene, carbohydrates, lipids and proteins that is used to produce a great variety of products. Half of them are of medical use, such as examination gloves, tubes, syringes, catheters, etc., but others are of general use, such as protective gloves, nipples for feeding babies, condoms and balloons.

During the past decade, latex allergy has become an increasing problem in occupational and public health, mainly due to the generalisation of protective measures against AIDS and hepatitis virus. The prevalence of latex allergy in the general population is less than 1% [1], but the occurrence of specific anti-latex IgE is higher (4–7%) [2]. However, there are risk groups where the prevalence of latex allergy is higher, as is the case of people who wear gloves at work, such as healthcare workers (5–15%) and people who have suffered repeated surgical operations like children with spina bifida (24–60%).

The protein content of latex is 2–3%. More than 50 NRL proteins, out of around 250, are able to bind IgE from latex-allergic patients in immunoblotting analysis [3–8]. Several of these proteins have been purified and characterised, and 11 of them have allergen nomenclature designations registered by WHO–IUIS (see Breiteneder and Scheiner [9] and Kurup and Fink [10] for reviews). The molecular characteristics and biological functions of these 11 latex allergens are shown in Table 6.1. The significance of these allergens as antigens is variable among the different risk groups [9, 11]. Most of them have been cloned and expressed in heterologous systems. Both purified native and recombinant allergens are becoming valuable tools for the diagnosis of latex allergy through immunoblotting and skin prick tests [12], and also for immunotherapy [13].

Other latex allergens, without registered nomenclature, include a class II chitinase, a class III chitinase/lysozyme and a triosephosphate isomerase [7, 8, 14].

Many proteins of plant foods and pollens share different degrees of homology with latex allergens, and can potentially be recognised by IgE of latex-allergic patients [15]. These plant proteins are good candidates to be responsible for cross-reactions between latex and plant foods or between latex and pollens. Table 6.2 lists these plant

**Table 6.1** WHO–IUIS nomenclature, molecular characteristics and physiological function of latex allergens

| Allergen | Trivial name | Function in latex | Molecular characteristics | Accession number |
|---|---|---|---|---|
| **Hev b 1** | Rubber elongation factor (REF) | Rubber biosynthesis: prenyltranferase cofactor | pI 5.04; 14.6 kDa; homotetramer 58 kDa | X56535; P15252 |
| **Hev b 2** | β-1,3-glucanase | Pathogenesis-related protein PR-2 | pI 9.5; 41.3 kDa; mature protein 35.1 kDa | U22147; P52407 |
| **Hev b 3** | Small rubber particle protein | Rubber biosynthesis: homologous to REF | pI 4.3–5; 23–27 kDa | AF051317; AJ223388 |
| **Hev b 4** | Microhelix component | ? | pI 4.5; 50–57 kDa; homodimer 100–110 kDa | |
| **Hev b 5** | Acidic protein | ? | pI 3.5; 16 kDa | U51361; U42640 |
| **Hev b 6.01** | Prohevein | Pathogenesis-related protein | pI 5.6; 20 kDa | M36986; P02877 |
| **Hev b 6.02** | Hevein | PR-4 | pI 4.9; 4.7 kDa | |
| **Hev b 6.03** | Prohevein *C*-terminal domain | Coagulation of latex | pI 6.4–7.7; 14 kDa | |
| **Hev b 7** | Patatin-like protein. Esterase? | Defence protein | pI 4.8; 43 kDa | AJ223038; U80598 |
| **Hev b 8** | Profilin | Structural protein | 15 kDa | AJ132397; AJ243325 |
| **Hev b 9** | Enolase | ? | 48 kDa | AJ132580; AJ132581 |
| **Hev b 10** | Mn-superoxide dismutase | ? | 23 kDa | AJ289158; AJ249148 |
| **Hev b 11** | Class I endochitinase | Pathogenesis-related PR-3 | 32 kDa | AJ238579 |

proteins and their counterpart latex allergens. The implications of most of these homologies between latex allergens and plant proteins for clinical relevant allergies requires further study, but it seems clear that hevein and its precursor prohevein are the main latex allergens implicated in cross reactivities with plant foods.

Hevein (Hev b 6.02) is a 4.7 kDa polypeptide of 43 amino acids, which is rich in cysteine and glycine, and binds chitin. Its fungicidal activity, and induction by wounding and infection has led to the proposal that it plays a defence function in the rubber tree. It is also involved in the coagulation of latex by forming bridges between rubber particles, which may be part of a defensive mechanism [16, 17].

Hevein is synthesised as a precursor of 20 kDa prohevein (Hev b 6.01), which is cleaved by proteolysis to produce the *N*-terminal hevein and a 14 kDa *C*-terminal domain of 144 amino acids, which is also a latex allergen (Hev b 6.03). The three-dimensional structure of hevein and its complex with *N*-acetylglucosamine oligosaccharides have been solved [18–20], and its structural scheme is shown in Plate 6.1. It is comprised of four-stranded β-sheets, five turns and a short *C*-terminal α-helix. Three aromatic

**Table 6.2** Plant proteins and latex allergens with homologous amino acid sequences

| Plant proteins | Latex allergens |
|---|---|
| **Class I chitinases**<br>Avocado (Prs a 1)<br>Chestnut (Cas s 5)<br>Banana (Mus a 1.1 y Mus a 1.2)<br>Green bean, cereals . . .<br>*Ficus benjamina*?<br>**Hevein-like antimicrobial peptides**<br>*Sambucus nigra* (elderderry)<br>*Beta vulgaris* (sugar beet)<br>*Amaranthus caudatus*<br>*Pharbitis nil*<br>**Prohevein-like**<br>Tobacco<br>*Brassica rapa* (turnip) (Bra r 2)<br>**Win proteins (proheveins)**<br>Potato<br>**Class IV chitinases**<br>Grapes, maize, sugar beet, yam, carrot . . . | **Prohevein** (Hev b 6.01)<br>**Hevein** (Hev b 6.02)<br>***C*-terminal domain** (Hev b 6.03)<br>**Class I chitinase**(Hev b 11) |
| **Profilins**<br>Pollens (Bet v 2, etc.)<br>Foods (Api g 4, Pru av 4, etc.) | **Profilin** (Hev b 8) |
| **Patatin**<br>Potato (Solt t 1) | **Patatin homologous** (Hev b 7) |
| **β-1,3-glucanases**<br>(banana, tomato, potato . . .) | **β-1,3-glucanase** (Hev b 2) |
| **Acidic protein**<br>Kiwi | **Acidic protein** (Hev b 5) |
| **Class II chitinases**<br>Barley, chestnut, avocado . . . | **Class II chitinase**<br>**Class I chitinase** (Hev b 11) |
| **Class III chitinases**<br>Papaya, rice, sugar beet . . . | **Hevamine** |

residues (tryptophans 21and 23, and tyrosine 30), as well as serine 19 and the loop between amino acids 13 and 15, seem to be essential for chitin and oligosaccharide binding. Several hevein molecules can bind to the same chitin molecule, a fact that may be related to its defensive role [21]. It must be emphasised that the main IgE-binding epitopes so far described are located in this chitin-binding region of hevein [22,23].

### 6.1.2 *Prevalence and clinical symptoms of the sensitisation to fruits in latex-allergic patients*

The first description of a patient with latex- and banana-associated allergy was at the beginning of the 1990s [24]. Soon after, there were some reports of patients showing simultaneous allergy to latex and several fruits [25–29]. In 1994, the existence of a *latex–fruit syndrome* was proposed, based on the clinical observation of an unexpected high rate of fruit immediate hypersensitivity in a group of 25 latex-allergic patients [30]. In this study, almost 50% of latex-allergic patients showed food allergy. Implicated fruits were mainly chestnut, avocado and banana; although kiwi, papaya and other foods were also involved. With respect to their clinical manifestations, half of the adverse reactions consisted of systemic anaphylaxis, thus demonstrating the clinical relevance of these associated sensitisations; and the other half varied between urticaria, angioedema and oral allergy syndrome.

Foods responsible for adverse reactions in a group of 50 latex-allergic patients, as well as their clinical manifestations, are summarised in Fig. 6.1. Among these 50 patients, a total of 72 food symptomatic sensitisations were diagnosed. Banana and avocado hypersensitivities were most frequent (28% of the 50 latex-allergic patients showed allergy to them), followed by chestnut (24%) and kiwi (20%) [31].

Although there are no close taxonomic relationships between the various plant foods implicated in this latex–fruit syndrome, its existence has been fully

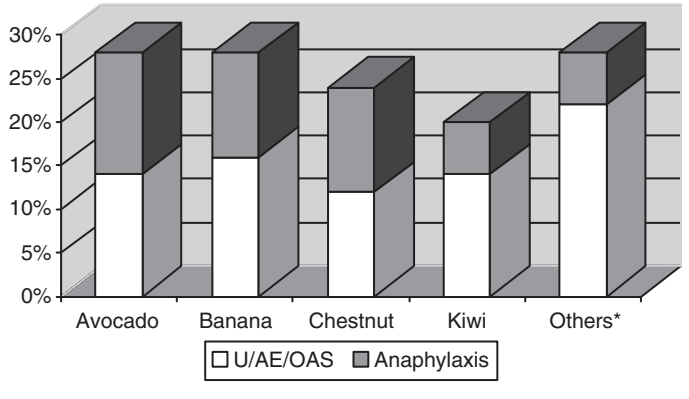


**Fig. 6.1** Relative frequency and clinical manifestations of food allergies in a group of 50 patients allergic to latex. U: urticaria; AE: angioedema; OAS: oral allergy syndrome.
*Including foods such as papaya, fig, passion fruit, *Prunoideae* fruits (peach, medlar, plum), potato, nuts and cereals.

confirmed by other authors from different countries [32–37]. A comparison of these studies leads to the following observations:

1. The proportion of latex-allergic patients who show associated food allergy varies from 21% to 58% among the studies considered (Table 6.3). This variability could be explained by differences in diagnostic criteria, both for latex allergy and food hypersensitivity. In this context, the criteria for diagnosing latex allergy are not standardised, and a gold standard is not available, and therefore latex-allergic patients are not similarly selected. Furthermore, food oral challenge tests have not been performed with these patients, leading to a probable over-diagnose of food allergies. However, differences in food consumption habits could also play a role. In any case, as usually observed when dealing with food allergy, the rate of food sensitisations among latex-allergic patients could be considerably higher, with many of them being asymptomatic [35].

2. As previously suspected, the type and proportion of food sensitivities associated with latex allergy vary among these studies [30]. This fact may be explained by differences in the nutritional habits between countries [30, 36]. As an example, chestnut and avocado allergies are less frequently diagnosed in Germany than in Spain, probably because these foods are less consumed in the former.

3. In the same way, the rate of food anaphylactic reactions varies between the studies, from 50% [31] to less than 5% [36] of recorded adverse food reactions. Again, differences in diagnostic criteria and food consumption habits could explain these figures. In fact, certain foods seem to be more prone to induce systemic anaphylaxis, such as banana, avocado, chestnut and kiwi [30–35]. Other foods, which are not very often associated with latex allergy, may also induce anaphylactic reactions, as for example fig, papaya and tomato. Other foods such as potato usually induce mild local reactions [35]. As frequently described with latex allergy, systemic anaphylaxis could be the initial manifestation of the food hypersensitivity.

**Table 6.3** Occurrence of food hypersensitivity among latex-allergic patients

| Reference | Country | No. of latex-allergic patients | % of food sensitisation/allergy (diagnostic criteria) |
|---|---|---|---|
| Blanco *et al*. 1994 | Spain | 25 | 52% (history + PPT) |
| Mäkinen-Kiljunen 1994 | Finland | 31 | 52% (history)<br>35% (PPT) |
| Lavaud *et al*. 1995 | France | 17 | 58% (history + SPT) |
| Delbourg *et al*. 1996 | France | 16 | 50% (history)<br>36% (SPT) |
| Beezhold *et al*. 1996 | Canada | 47 | 36% (history + SPT)<br>70% (SPT) |
| Blanco 1997 | Spain | 50 | 46% (history + PPT) |
| Brehler *et al*. 1997 | Germany | 136 | 43% (history)<br>69% (IgE)<br>14% (history + IgE) |
| Kim & Hussain 1999 | USA | 137 | 21% (history) |

PPT: prick by prick test; SPT: skin prick test; IgE: food-specific IgE determination.

### 6.1.3 In vitro *latex–fruit cross-reactivity*

Early studies using different methods, such as RAST inhibition, clearly demonstrated cross-reactivity between latex and various fruits [30, 32, 35, 36, 38, 39]. Several fruit antigens with common epitopes with latex allergens were identified by immunoblot inhibition assays [40, 41]. Moreover, the main uncharacterised allergens responsible for cross-reactivity of avocado and banana with latex were found to be 30–37 kDa in size [33, 34].

Chitinases are one of the most abundant protein types described and characterised in chestnut seeds [42], one of the foods more often involved in the latex–fruit syndrome in Spain. Chestnut class I chitinases have a molecular weight of around 30 kDa, and contain an *N*-terminal region with more than 60% sequence identity with the latex allergen hevein (Hev b 6.02). The similarity in their molecular weights, to those of the avocado and banana allergens, and the presence of an hevein domain indicate that fruit class I chitinases may be the major allergens implicated in the latex–fruit syndrome.

## 6.2 Plant chitinases

Chitinase enzymes are widely distributed among living organisms. They are present in organisms that contain chitin, such as arthropods, crustaceans and fungi, as well as in others without chitin such as bacteria, higher plants and vertebrates. The main natural substrate of chitinases is chitin, which is the second most abundant polysaccharide in nature after cellulose. Chitin is a linear polymer of *N*-acetylglucosamine (GlcNAc) with β-1,4 linkages, and is a major structural component of fungal cell wall, arthropod cuticles and crustacean exoskeletons.

The biological function of chitinases in invertebrate organisms is the hydrolysis of chitin in cell walls and cuticles during growth and moulting processes [43, 44]. In organisms lacking chitin, it seems that the major role of chitinases is defence against predators.

Plant chitinases are mostly endochitinases, which randomly hydrolyse internal β-1,4 linkages of chitin and of GlcNAc-containing oligosaccharides. Some of them also show lysozyme activity, hydrolysing the β-1,4 linkages between *N*-acetyl-muramic acid and GlcNAc residues of bacterial pectidoglycan.

Plant chitinases have been classified into six classes (I–VI) based on homologies in their primary structures [45, 46] and consensus sequences [47]. Figure 6.2 shows a schematic representation of their structures and homology relationships. Class I chitinases have a molecular weight of around 33 kDa and share an *N*-terminal sequence, the hevein-like domain, of approximately 40 amino acid residues with almost 70% sequence identity with the latex hevein. This *N*-terminal domain is linked to the catalytic domain, where the active center is located, through a hinge rich in glycine and proline or hydroxyproline residues. Other chitinases with one or two hevein domains are placed in classes IV and V, respectively. Class II chitinases and the catalytic domain of class IV chitinases are homologous to the catalytic domain of class I chitinases (with amino acid deletions in the latter case). Class III chitinases also show lysozyme activity. Class VI chitinases are not homologous to the other five

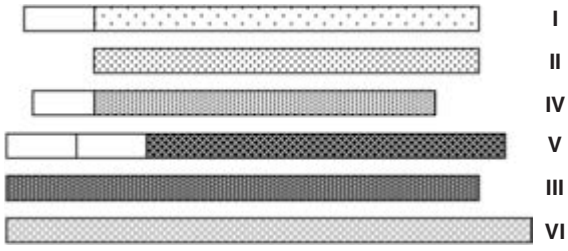


**Fig. 6.2** Structures and homology relationships of plant chitinases. I–VI are the main chitinase classes. White areas represent hevein domains and hatched areas represent catalytic domains with different degrees of sequence identity.

classes but have some sequence identity to bacterial exochitinases. All of these chitinase classes are included in family 19 of glycosyl hydrolases, except the class III enzymes that belong to family 18 [48]. The two glycosyl families differ in structure and catalytic mechanism [49]. Family 19 enzymes have a core structure of α-helices and display an inverting mechanism (single displacement), while family 18 members are essentially $(\alpha\beta)_8$ barrels that operate by a retaining mechanism (double displacement).

Several lines of evidence support the suggestion that the main biological function of plant chitinases is defence against predators: (a) they are induced by infection and also by ethylene and salicylic acid, two plant hormones implicated in defence responses of plants [50–54]; (b) *in vitro*, they are toxic to fungi, although in some cases only in combination with β-1,3-glucanases [55–57]; (c) transgenic plants expressing chitinase genes have reduced susceptibility to fungal infection [58–62]. In fact, chitinases are included in different classes of the PR (pathogenesis-related) proteins, so called because they are induced by wounding, infection or pest attack, and may be toxic to pests and pathogens (see van Loon and van Strien [63], for a review of the nomenclature and function of PR proteins).

Furthermore, several types of chitinases, among other defence proteins, contribute to the plant *immune* mechanism known as *systemic acquired resistance* (SAR) [53, 64]. In this way, some of the most recent agricultural fungicides that are activators of SAR, like probenazole or benzothiadiazole (BTH) can induce the expression of chitinases.

Plant chitinases may also have other physiological functions. They are involved in the control of embryo development. In carrot, it has been shown that they are able to rescue a somatic embryo mutant [65, 66]. Some chitinases have also been implicated in the defence of plants against abiotic stresses such us cold and freezing. Winter rye accumulates cold-responsive chitinases that show antifreeze activity when expressed in *E. coli* [67]. Chitinases also regulate the activity of nodulation (*nod*) factors during infection of Leguminoseae plants by the symbiotic nitrogen-fixing bacteria *Rhizobium spp*. *Nod* factors are lipochitooligosaccharides with β-1,4 linked *N*-acetylglucosamine subunits. These molecules, produced by the bacteria, act as plant-growth regulators that elicit morphogenetic responses in the plant (host) that favour nodule development [68]. It has been found that some chitinases, mainly of class II, are induced by *nod* factors which are then hydrolysed and inactivated by them [69]. It has been postulated that

through this hydrolysis, plant chitinases may regulate the timing of *nod* activity and even the specificity of the bacteria–host plant interaction [70]. Moreover, different chitinase classes have distinct substrate specificities towards modified *nod* factors *in vitro* [71].

## 6.3 The panallergens of the latex–fruit syndrome are class I chitinases

### 6.3.1 *Chestnut, avocado and banana class I chitinases as major allergens of the latex–fruit syndrome*

Different authors have identified class I chitinases from avocado [72–75], chestnut [72, 75] and banana [72, 77], three of the fruits most frequently related to the latex–fruit syndrome as the allergens responsible for this cross-reactivity [76].

The IgE-binding proteins shown in Fig. 6.3 were purified and characterised. Like class I chitinases, they show chitinase activity, bind anti-chitinase IgG antibodies, and their *N*-terminal amino acid sequences are homologous to the latex hevein. Other proteins of avocado and chestnut which have lower molecular weights, react with the chitinase antibodies but do not bind IgE of latex–fruit-allergic patients have been identified as class II chitinases [72].

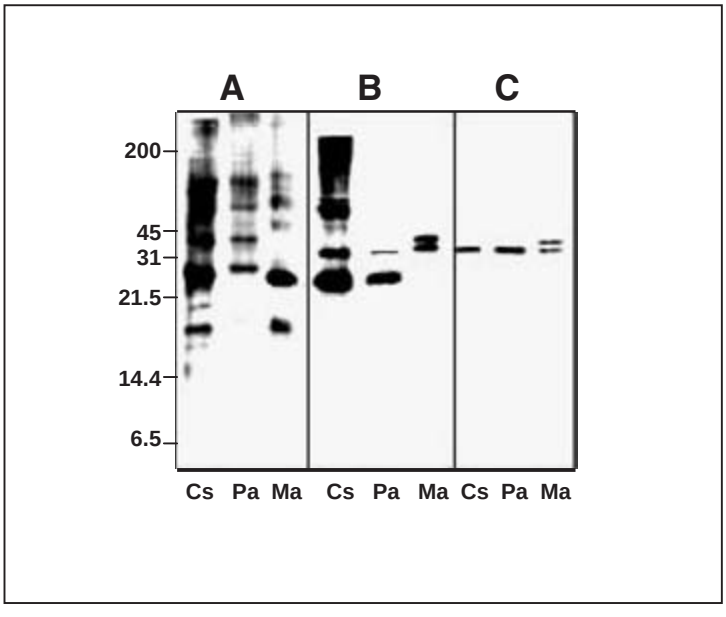


**Fig. 6.3** IgE-binding proteins from chestnut (*Castanea sativa*: Cs), avocado (*Persea americana*: Pa) and banana (*Musa acuminata*: Ma). Protein extracts from the corresponding fruits were separated by SDS-PAGE. Replica gels were stained with Coomassie Blue **(A)** or electrotransferred to PDVF membranes and immunodetected with monospecific polyclonal anti-chitinase antibodies **(B)** or with a pool of sera from latex–fruit-allergic patients **(C)**.

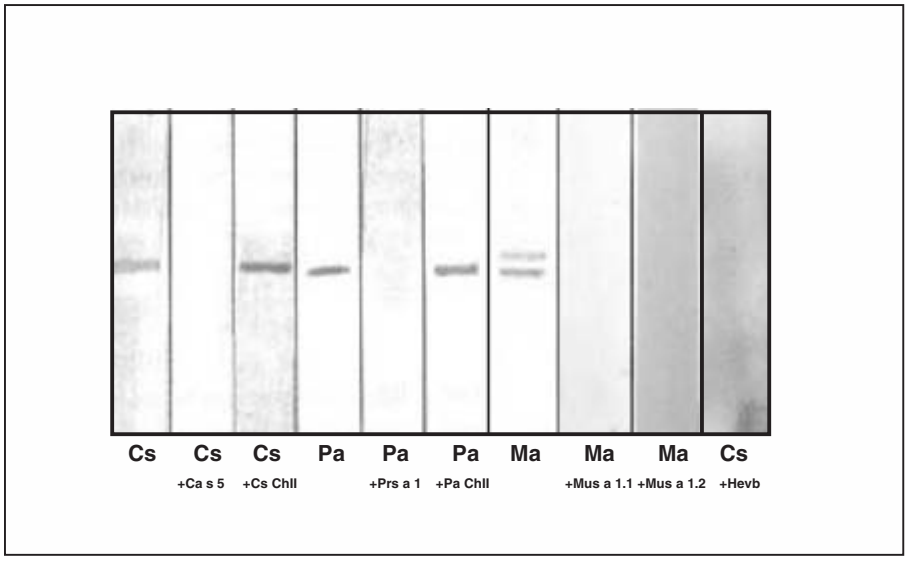


**Fig. 6.4** Immunoblot of inhibition assays using purified class I and II chitinases as inhibitors. Crude protein extracts from chestnut (Cs), avocado (Pa) or banana (Ma) separated by SDS-PAGE and electrotransfered to PVDF membranes were immunodetected with a pool of sera from latex–fruit-allergic patients preincubated with buffer or with purified class I chitinases from chestnut (+Cas s 5) avocado (+Prs a 1) or banana (+Mus a 1.1 and +Mus a 1.2), as well as class II chitinases from chestnut (+CsChII) or avocado (+PaChII) and a crude latex protein extract (+Hevb).

In immunoinhibition assays with purified proteins such us those shown in Fig. 6.4, class I chitinases, but not class II chitinases which lack the hevein domain, completely inhibited the IgE binding of crude protein extracts from chestnut, avocado and banana. A latex protein extract also inhibited the IgE binding of the chestnut extract.

In RAST inhibition assays, class I chitinases inhibited nearly 90% of the IgE binding of crude extracts, while only around 40% of inhibition was reached with class II chitinases [75, 79]. Moreover, purified hevein completely inhibited the IgE-binding capacity of avocado protein extracts, when sera of latex- and avocado-allergic patients were used [74, 78].

Skin prick tests with the purified chestnut, avocado and banana class I chitinases were positive in more than 50% of the patients studied, while the figures were much lower for class II chitinases [75, 77].

### 6.3.2 *The hevein domain of class I chitinases is mainly responsible for their IgE-binding capacity but the catalytic domain may also have an important role*

The *in vitro* and *in vivo* experiments described above point to class I chitinases as major allergens related to latex allergy in chestnut, avocado and banana. Their hevein domains play a crucial role in the IgE-binding capacity of these proteins, and hevein seems to be the cross-reacting allergen in latex.

The amino acid sequences of the hevein-like domains of class I allergenic chitinases from avocado, chestnut and banana are aligned with that of latex hevein in Fig. 6.5. The high degree of sequence identity between the hevein domains of these proteins and the latex hevein must account for the presence of common epitopes, which are recognised by IgE antibodies of latex–fruit-allergic patients.

Figure 6.5 also shows the amino acid sequence of a latex class I chitinase (Hev b 11) that has been characterised and expressed as recombinant product, fused to maltose-binding protein [79]. Its role in the sensitisation of latex-allergic patients to fruits needs to clarified. It is surprising that only 22% of the latex-allergic patients studied had IgE specific to Hev b 11. Inhibition of hevein IgE-binding by Hev b 11was also low.

The amino acid sequence of a tobacco class II chitinase is also shown in Fig. 6.5 as representative of this type of chitinase that lacks the hevein domain, but shows a high degree of sequence identity with the catalytic domain of class I chitinases. The presence of allergenic class I and class II chitinases in latex may imply that other epitopes, besides those of the hevein domain of chitinases, can contribute to fruit hypersensitivity in latex-allergic patients.


**Fig. 6.5** **A.** Amino acid sequence alignment of latex hevein and the hevein domains of fruit allergenic and latex class I chitinases. **B.** Amino acid sequence comparison of the catalytic domains of fruit allergenic and latex class I chitinases and tobacco class II chitinase. Hev b 6.02: Latex hevein (accesion number P02877); Prs a 1: Avocado class I chitinase (Z78202); Cas s 5: Chestnut class I chitinase (U48687); Mus a 1.1: Banana class I chitinase (AF001524); Hev b 11: Latex class I chitinase (AJ238579); NT chII: Tobacco class II chitinase (X51425).

The role of the hevein-like and catalytic domains of class I chitinases in their IgE-binding capacity has been studied more directly [80] by using a chestnut class I chitinase (Cas s 5) cDNA clone [81]. The complete mature protein clone (rCas s 5) and a deletion mutant (rCAT), which only includes the catalytic domain, were expressed in the yeast *Pichia pastoris* and the recombinant products purified. Both the recombinant proteins showed chitinase activity and were recognised by anti-chitinase antibodies. In IgE-immunodetection, only rCas s 5, but not rCAT, bound IgE from the sera of latex–fruit-allergic patients. Moreover, the latex hevein almost completely inhibited this IgE binding. It can be concluded from these results that the main IgE epitopes of class I chitinases involved in the latex–fruit syndrome are located in their hevein domain, and that latex hevein is the cross-reactive latex allergen.

However, it should be pointed out that the immunodetection and immunoinhibition assays described above were done in the presence of denaturating agents. In RAST (CAP) experiments, which were carried out in native conditions, chestnut class I chitinases (natural and recombinant) inhibited more than 90% of the IgE binding of the chestnut protein extract. The fact that both rCAT and native chestnut class II chitinase inhibited this IgE binding at levels approaching 60% suggests that there are also IgE epitopes in the catalytic domain, probably conformational ones, that are not detectable under denaturing conditions.

### 6.3.3 *Class I chitinases as panallergens of the latex–fruit syndrome*

As discussed above, chitinases are widely distributed in plant foods. The role of class I chitinases as potential panallergens implicated in the latex–fruit syndrome has recently been studied [82]. Protein extracts from 19 plant foods, showing different relationships with latex allergy, including fruits, legumes, vegetables, nuts and cereals, were analysed. The presence of 30–45 kDa proteins, which reacted with anti-chitinase antibodies and bound to IgE from sera of latex–fruit-allergic patients, was detected not only in foods previously related with the syndrome (kiwi, chirimoya, passion fruit, papaya, mango and tomato), but also in other foods not usually associated with it. The IgE binding of these proteins was inhibited by a latex protein extract and purified allergenic class I avocado chitinase (Prs a 1). These results indicate that putative class I chitinases (or closely related allergens) are the main panallergens implicated in the latex–fruit syndrome. The fact that these proteins do not bind the IgE of patients who have latex allergy but are not sensitised to fruits, suggests different patterns of sensitisation for the two types of allergic patients.

An association between allergies to latex and the ornamental plant *Ficus benjamina* has been described recently [83]. Once again, the cross-reactive allergen is latex hevein, and its counterpart in *F. benjamina* is an acidic protein with a molecular weight of about 45 kDa and an *N*-terminal hevein-like domain [84].

### 6.3.4 *Heat inactivation, ethylene induction and digestive behaviour of plant allergenic class I chitinases*

Raw plant foods, such as fruits, have mainly been related to the latex–fruit syndrome. In contrast, those foods that are subjected to home or industrial heat treatments are

rarely associated with latex allergy, even if they contain allergenic class I chitinases. This fact can be explained by the heat inactivation of these allergens.

The heat inactivation of class I allergenic chitinases has been investigated using green bean as a model system [85]. Green bean is a legume which is widely consumed after cooking and is not associated with the latex–fruit syndrome, but has an ethylene-induced class I chitinase [50]. A class I chitinase purified from green beans bound IgE from latex–fruit-allergic patients' sera, and provoked skin prick reactions in seven out of eight patients analysed. It has around 75% sequence identity with the allergenic avocado and chestnut class I chitinases, and is fully cross-reactive with the avocado protein in immunoinhibition assays. After a heat treatment of 15 minutes at 100°C, both the green bean and the avocado class I chitinases lost their *in vitro* IgE-binding capacity, and did not provoke positive skin prick tests in the same patients who reacted to the untreated allergens. This inactivation was also observed in commercial canned and frozen green beans, as well as in chestnut derivatives, such us puree and syrup, all of which have undergone a heat treatment during their industrial processing (Sánchez-Monge *et al*. unpublished).

The amount of green bean class I chitinase was effectively increased by ethylene treatment. Ethylene is a plant hormone that is used to hasten ripening of fruits such as apple, avocado, banana and tomato. This result could be of clinical relevance because ethylene treatment can strongly increase the allergen content of these fruits.

Stability to proteolytic digestion has been proposed as a general characteristic of food allergens [86]. There are only two inconclusive studies on the stability of plant class I chitinases using simulated gastric fluid. In one of them [87], putative class I chitinases, after a few minutes of treatment, were not detected by protein staining or by immunodetection with latex–fruit-allergic patients' sera. Posch *et al*. [74] showed that the avocado allergen (Prs a 1) was rapidly hydrolysed, but the 4–6 kDa molecular weight peptides arising from this were stable even after 60 minutes of treatment and completely inhibited the avocado-IgE specific binding of sera from avocado and latex-allergic patients. It is probable that proteolytically stable peptides with molecular weights similar to that of hevein contain the IgE-binding epitopes responsible for this cross-reactivity.

## 6.4 Other plant proteins with hevein-like domains that could be implicated in plant–latex cross-reactivities

In addition to class I chitinases, class IV and V enzymes and other plant proteins have domains homologous to latex hevein. Among them are the turnip and tobacco pro-heveins, as well as tomato and potato Win (wound-induced) proteins, which also have *C*-terminal domains highly similar to that of the hevein precursor, prohevein.

Wheat germ agglutinin has four hevein domains in tandem. Other plant lectins, such us those from *Pharbitis nil*, elderberry, stinging nettle, mistletoe and amaranthus, are peptides of 30–48 amino acids homologous to latex hevein (Fig. 6.6).

The biological function of these chitin-binding proteins is thought to be defensive [88]. Most of them are toxic to fungi and, in some cases, antibacterial and insect anti-feeding activities have been described. The toxicity of chitinases may be derived from

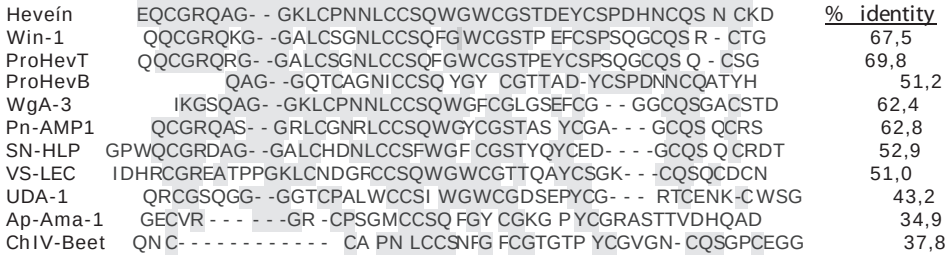


**Fig. 6.6** Amino acid sequence alignment of latex hevein and the hevein domains of different plant proteins. Hevein: Latex hevein (accesion number P02877); Win-1: *Solanum tuberosum* (potato) wound-induced protein 1 (P09761); ProHevT: *Nicotiana tabacum* (tobacco) prohevein-like defense protein CBP20 (S72452); ProHevB: *Brassica rapa* (turnip) prohevein-like allergenic protein (P81729); WgA-3: *Triticum aestivum* (wheat) germ agglutinin domain 3 (P10968); Pn-AMP1: *Pharbitis nil*: antimicrobial protein 1 (P81591); SN-HLP: *Sambrucus nigra* (elderderry) hevein-like protein (AF074385); VS-LEC: *Viscum album* (mistletoe) lectin (P81859); UDA-1: *Urtica dioica* (nettle) agglutitin domain 1 (X13497); Ap-Ama-1: *Amaranthus caudatus* (amaranth) antimicrobial peptide 1 (P27275); ChIV-Beet: *Beta vulgaris* (sugar beet) class IV chitinase (S46536).

the hydrolysis of chitin. However, no enzymatic function has been described for other chitin-binding proteins, and the molecular mechanism of their toxicity is not well understood. Low molecular weight lectins can pass through the cell walls of fungi and affect their morphogenesis [89]. This mode of action is supported by the fact that the larger lectins of the Gramineae are not toxic to fungi, and their insect antifeeding properties may be due to chitin binding in the peritrophic membranes of the insect gut [90]. In some cases, the chitin-binding capacity of these peptides is not essential for their defensive function, since they are toxic to the fungi which do not contain chitin. The fact that they induce pores in cellular membranes, with loss of cellular components and cell lysis [91], suggests a toxic mechanism similar to that of other antimicrobial peptides, like thionins and lipid transfer proteins (LTPs) [92, 93]. It is noteworthy that, as mentioned above, the levels of these defensive proteins are increased by certain agricultural treatments.

The hevein domain of these proteins might be recognised by IgE antibodies of latex-allergic patients, since their similarity with latex hevein is quite high (up to 70% of sequence identity). There are only a limited number of studies about these possible cross-reactivities, that are described below.

In immunoblotting analysis, wheat germ agglutinin (WGA) binds to IgE of sera from latex-allergic patients [23]. The binding was fully inhibited by a latex protein extract, but WGA only exerted weak inhibition of the binding of latex hevein and prohevein IgE. The almost complete sequence identity between one of the hevein epitopes and some regions of WGA probably explains this fact. However, in another study [72], the sera of latex–fruit-allergic patients did not recognise WGA, and the agglutinin did not inhibit the IgE binding of the class I chestnut chitinase. As discussed above, it is possible that different IgE-binding epitopes in latex hevein are differentially recognised by sera from latex and latex–fruit-allergic patients.

Hänninen *et al*. [94] showed that a turnip prohevein binds IgE of sera from more than 80% of latex-allergic patients. This binding was 80% inhibited by hevein, and there was a complete inhibition by latex prohevein. The turnip prohevein is not

present in healthy plants, but it is induced by ethylene and salicylic treatment. The cross-reactivity of a tobacco prohevein has been also studied by Hänninen *et al*. [95]. Sera from 73% of latex-allergic patients showed IgE binding to this prohevein, with the IgE binding being inhibited by purified latex prohevein. Some of the sera that recognised the tobacco prohevein had specific IgE against prohevein, but not against hevein. In this way, the high degrees of sequence identity between the *C*-terminal domains of turnip and tobacco proheveins, and between the potato and tomato Win-proteins and latex prohevein may account for the cross-reactivity of these plant species and latex. Some of the sequential B epitopes of the latex prohevein have been located in its *C*-terminal domain [22–23].

## 6.5 Final remarks

Plant class I chitinases have been identified as major allergens responsible for the latex–plant food cross-reactivities known as latex–fruit syndrome. Their *N*-terminal regions, which are homologous to the latex allergen hevein, seem to contain main epitopes recognised by IgE of the sera of latex-allergic patients.

Due to the heat inactivation of these allergens, only raw consumed foods, mainly fruits, are associated with the syndrome. The amount of these allergens in fruits may be increased by ethylene treatments used to hasten fruit ripening.

cDNA clones for some of these allergenic chitinases and the corresponding recombinant proteins have been obtained, and will be useful for the diagnosis and potential immunotherapy of latex-related allergy. Moreover, by protein engineering and modelling the three-dimensional structures, it may be possible to locate the major IgE epitopes and to obtain hypoallergenic variants.

## Acknowledgements

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## References

1. Poley, G.E. & Slater, J.E. (2000) Latex allergy. *J Allergy Clin Immunol* **105**, 1054–1062.
2. Merret, T.G., Merret, J. & Kekwik, R. (1999) The prevalence of immunoglobulin E antibodies to the proteins of rubber (*Hevea brasiliensis*) latex and grass (*Phleum pratense*) pollen in sera of British blood donors. *Clin Exp Allergy* **29**, 1572–1578.
3. Slater, J.E. & Chabra, S. (1992) Latex antigens. *J Allergy Clin Immunol* **89**, 673–678.
4. Mäkinen-Kiljunen, S., Turjanmaa, K., Palosuo, T. & Reunala, T. (1992) Characterization of latex antigens and allergens in surgical goves and natural rubber by immunoelectrophoretic methods. *J Allergy Clin Immunol* **90**, 230–235.

5. Alenius, H., Palosuo, T., Kelly, K. *et al*. (1993) IgE reactivity to 14 kD and 27 kD natural rubber proteins in latex-allergic children with spina bifida and other congenital anormalies. *Int Arch Allergy Immunol* **102**, 61–66.
6. Kurup, V.P., Alenius, A., Kelly, K.J., Castillo, L. & Fink, J.N. (1996) A two-dimensional electrophoretic analysis of latex peptides reacting with IgE and IgG antibodies from patients with latex allergy. *Int Arch Allergy Immunol* **109**, 58–67.
7. Posch, A., Chen, Z., Wheeler, C., Dunn, M.J., Raulf-Heimsoth, M. & Baur, X. (1997) Characterization and identification of latex allergens by two-dimensional electrophoresis and protein microsequencing. *J Allergy Clin Immunol* **99**, 385–395.
8. Yagami, T., Sato, M., Nakamura, A. *et al*. (1998) Plant defense-related enzymes as latex antigens. *J Allergy Clin Immunol* **101**, 379–385.
9. Breiteneder, H. & Scheiner, O. (1998) Molecular and immunological characteristics of latex allergens. *Int Arch Allergy Immunol* **116**, 83–92.
10. Kurup, V.P. & Fink, J.N. (2001) The spectrum of immunological sensitization in latex allergy. *Allergy* **56**, 2–12.
11. Kurup, V.P., Yeang, H.Y., Sussman, G.L. *et al*. (2000) Detection of immunoglobulin antibodies in the sera of patients using purified latex allergens. *Clin Exp Allergy* **30**, 359–369.
12. Yip, L., Hickey, V., Wagner, B. *et al*. (2000) Skin prick test reactivity to recombinant latex allergens. *Int Arch Allergy Immunol* **121**, 292–299.
13. Breiteneder, H., Krebitz, M., Wiedermann, U. *et al*. (2001) Rapid production of recombinant allergens in *Nicotiana benthamiana* and their impact on diagnosis and therapy. *Int Arch Allergy Immunol* **124**, 48–50.
14. Yagami, T., Sato, M., Nakamura, A. & Shono, M. (1995) One of the rubber latex allergens is a lysozyme. *J Allergy Clin Immunol* **96**, 677–686.
15. Salcedo, G., Díaz-Perales, A. & Sánchez-Monge, R. (2001) The role of plant panallergens in sensitisation to natural rubber latex. *Curr Opin Allergy Clin Immunol* **1**, 177–183.
16. Van Parijs, J., Broekaert, W.F., Goldstein, I.J. & Peumans, W.J. (1991) Hevein: an antifungal protein from rubber tree (*Hevea brasiliensis*) latex. *Planta* **183**, 258–264.
17. Gidrol, X., Lee, H.I., Tan, H.L. & Kush, A. (1994) Hevein, a lectin-like protein from *Hevea brasiliensis* (rubber tree) is involved in the coagulation of latex. *J Biol Chem* **269**, 9278–9283.
18. Rodriguez-Romero, A., Ravinchandran, K.G. & Soriano-García, M. (1991) Crystal structure of hevein at 2.8 Å resolution. *FEBS Lett* **291**, 307–309.
19. Asensio, J.L., Cañada, F.J., Bruix, M., Rodriguez-Romero, A. & Jimenez-Barbero, J. (1995) The interaction of hevein with N-acetylglucosamine-containing oligosaccharides. Solution structure of hevein complexed to chitobiose. *Eur J Biochem* **230**, 621–633.
20. Asensio, J.L., Cañada, F.J., Bruix, M. *et al*. (1998) NMR investigations of protein–carbohydrate interactions: refined three-dimensional structure of complex between hevein and methyl beta-chitobioside. *Glycobiology* **8**, 569–577.
21. Asensio, J.L., Cañada, F.J., Siebert, H.C. *et al*. (2000) Structural basis for chitin recognition by defense proteins: GlcNAc residues are bounded in a multivalent fashion by extended binding sites in hevein domains. *Chem Biol* **7**, 529–543.
22. Barnerjee, B., Wang, X., Kelly, K.J., Fink, J.N., Sussman, G.L. & Kurup, V.P. (1997) IgE from latex-allergic patients binds to cloned and expressed B cell epitopes of prohevein. *J Immunol* **159**, 5724–5732.
23. Beezhold, D.H., Kostyal, D.A. & Sussman, G.L. (1997) IgE epitope analysis of the hevein preprotein; a major latex allergen. *Clin Exp Immunol* **108**, 114–121.
24. M'Raihi, L., Charpin, D., Pons, A., Bougrand, P. & Vervloet, D. (1991) Cross-reactivity between latex and banana. *J Allergy Clin Immunol* **87**, 129–130.
25. Lavaud, F., Cossart, C., Reiter, V., Bernard, J., Deltour, G. & Holmquist, I. (1992) Latex allergy in patient with allergy to fruit [Letter]. *Lancet* **339**, 492–493.
26. Ceuppens, J.L., Van Durme, P. & Dooms-Goossens, A. (1992) Latex allergy in patient with allergy to fruit [Letter]. *Lancet* **339**, 493.
27. de Corres, L.F., Moneo, I., Muñoz, D. *et al*. (1993) Sensitization from chestnuts and bananas in patients with urticaria and anaphylaxis from contact with latex. *Ann Allergy* **70**, 35–39.
28. Rodríguez, M., Vega, F., García, M.T. *et al*. (1993) Hypersensitivity to latex, chestnut, and banana. *Ann Allergy* **70**, 31–34.
29. Añíbarro, B., García-Ara, M.C. & Pascual, C. (1993) Associated sensitization to latex and chestnut. *Allergy* **70**, 130–131.
30. Blanco, C., Carrillo, T., Castillo, R., Quiralte, J. & Cuevas, M. (1994) Latex allergy: clinical features and cross-reactivity with fruits. *Ann Allergy* **73**, 309–314.
31. Blanco, C. (1997) Alergia al látex. Tesis Doctoral. Universidad de Las Palmas de Gran Canaria.

32. Mäkinen-Kiljunen, S. (1994) Banana allergy in patients with immediate-type hypersensitivity to natural rubber latex: characterization of cross-reacting antibodies and allergens. *J Allergy Clin Immunol* **93**, 990–996.
33. Lavaud, F., Prevost, A., Cossart, C., Guerin, L., Bernard, J. & Kochman, S. (1995) Allergy to latex, avocado pear, and banana: evidence for a 30 kD antigen in immunoblotting. *J Allergy Clin Immunol* **95**, 557–564.
34. Delbourg, M.F., Guilloux, L., Moneret-Vautrin, D.A. & Ville, G. (1996) Hypersensitivity to banana in latex-allergic patients. Identification of two major banana allergens of 33 and 37 kD. *Ann Allergy Asthma Immunol* **76**, 321–326.
35. Beezhold, D.H., Sussman, G.L., Liss, G.M. & Chang, N.S. (1996) Latex allergy can induce clinical reactions to specific foods. *Clin Exp Allergy* **26**, 416–422.
36. Brehler, R., Theissen, U., Mohr, C. & Luger, T. (1997) Latex–fruit syndrome: frequency of cross-reacting IgE antibodies. *Allergy* **52**, 404–410.
37. Kim, K.T. & Hussain, H. (1999) Prevalence of food allergy in 137 patients. *Allergy Asthma Proc* **20**, 95–97.
38. Ross, B.D., McCullough, J. & Owbny, D.R. (1992) Partial cross-reactivity between latex and banana allergens. *J Allergy Clin Immunol* **90**, 409–410.
39. Ahlroth, M., Alenius, H., Turjanmaa, K., Mäkinen-Kijunen, S., Reunala, T. & Palosuo, T. (1995) Cross reacting allergen in natural rubber latex and avocado. *J Allergy Clin Immunol* **96**, 167–173.
40. Alenius, H., Mäkinen-Kiljunen, S., Ahlroth, M., Turjanmaa, K., Reunala, T. & Polosuo, T. (1996) Cross-reactivity between allergens in natural rubber latex and banana studied by immunoblot inhibition. *Clin Exp Allergy* **26**, 341–348.
41. Moller, M., Kayma, M., Vieluf, D., Paschke, A. & Steinhart, H. (1998) Determination and characterization of cross-reacting allergens in latex, avocado, banana and kiwi fruit. *Allergy* **53**, 289–296.
42. Collada, C., Casado, R., Fraile, A. & Aragoncillo, C. (1992) Basic endochitinases are major proteins in *Castanea sativa* cotyledons. *Plant Physiol* **100**, 778–783.
43. Kramer, K.J. & Koga, D. (1986) Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochem* **16**, 851–877
44. Gooday, G.W., Humphreys, A.M. & Mcintosh, W.H. (1986) Roles of chitinases in fungal growth. In: *Chitin in Nature and Technology*, Muzzareli, R.A., Jeunniauz, C. & Gooday, G.W. (eds). Plenum Press, New York, pp. 83–91.
45. Meins, F., Fritig, B., Linthorst, H.J.M., Mikkelsen, J.D., Neuhaus, J.M. & Ryals, J. (1994) Plant chitinase genes. *Plant Mol Biol Rep* **12**, S22–S28.
46. Neuhaus, J.M., Fritig, V., Linthorst, H.J.M., Meins, F., Mikkelsen, J.D. & Ryals, J. (1996) A revised nomenclature for chitinase genes. *Plant Mol Biol Rep* **14**, 102–104.
47. Levorson, J. & Chlan, C.A. (1997) Plant chitinase consensus sequences. *Plant Mol Biol Rep* **15**, 122–133.
48. Henrissat, B. & Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **293**, 781–788.
49. Robertus, J.D. & Monzingo, A.F. (1999) The structure and action of chitinases. In: *Chitin and Chitinases*, Jolles, P. & Muzzarelli, R.A.A. (eds). Birkhauser Verlag, Basel, Switzerland, pp. 125–135.
50. Broglie, K.E., Gaynor, J.J. & Broglie, R.M. (1986) Ethylene gene expresssion: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. *Proc Nat Acad Sci USA* **83**, 6820–6824.
51. Lawton, K., Ward, E., Payne, G., Moyer, M. & Ryals, J. (1992) Acidic and basic class III chitinases mRNA accumulation in response to TMV infection of tobacco. *Plant Mol Biol* **19**, 735–743.
52. Jung, J.L., Fritig, B. & Hahne, G. (1993) Sunflower (*Helianthus annuus* L.) pathogenesis related proteins. Induction by aspirin (acetylsalicylic acid) and characterization. *Plant Physiol* **101**, 873–880.
53. Kästner, B., Tenhaken, R. & Kaus, H. (1998) Chitinase in cucumber hypocotyls is induced by germinating fungal spores and by fungal elicitor in synergism with inducers of adquired resistance. *Plant J* **13**, 447–454.
54. Ji, C., Norton, R.A., Wicklow, D.T. & Dowd, P.F. (2000) Isoform patterns of chitinase and β-1, 3-glucanase in maturing corn kernels (*Zea mays* L.) associated with *Aspergillus flavus* milk stage infection. *J Agric Food Chem* **48**, 507–511.
55. Shlumbaum, A., Mauch, F., Vogel, U. & Boller, T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**, 365–367.
56. Broekaert, W.K., Parijs, J.V., Leyns, F., Joos, H. & Peumans, W.J. (1986) Comparison of some molecular, enzymatic, and antifungal properties of chitinases from tomato, apple, tobacco and wheat. *Physiol Mol Plant Pathol* **33**, 319–331.
57. Mauch, F., Maunch-Mani, B. & Boller, T. (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combination of chitinase an beta-1,3-glucanase. *Plant Physiol* **88**, 936–942.
58. Broglie, K.E., Chet, I., Holliday, M. *et al*. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254**, 4308–4314.

59. Vierheilig, H., Alt, M., Neuhaus, J.M., Boller, T. & Wiemken, A. (1993) Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorhizal symbiont *Glomus mosseae*. *Mol Plant Microbe Interact* **6**, 261–264.
60. Jach, G., Görmhardt, B., Mundy, J. *et al*. (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant J* **8**, 97–109.
61. Grisson, R., Grezes-Besset, V., Scheider, M. *et al*. (1996) Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing chimeric chitinase gene. *Nature Biotechnol* **14**, 643–646.
62. Suarez, V., Staehelin, C., Arango, R., Holtorf, H., Hofsteenge, J. & Meins, F. Jr. (2001) Substrate specificity and antifungal activity of recombinant tobacco class I chitinases. *Plant Mol Biol* **45**, 609–618.
63. Van Loon, L.C. & van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1. *Physiol Mol Plant Pathol* **55**, 85–97.
64. Ward, E.R., Ukness, S.J., Willians, S.C. *et al*. (1991) Coordinate gene activity in tobacco to agent that induce systemic acquired resistance. *Plant Cell* **3**, 1085–1094.
65. De Jong, A.J., Cordewener, J., Schiavo, F.L. *et al*. (1992) A carrot somatic embryo mutant is rescued by chitinase. *Plant Cell* **4**, 425–433.
66. Kragh, K.M., Hendriks, T., de Jong, A.J. *et al*. (1996) Characterization of chitinases able to rescue somatic embryos of the temperature-sensitve carrot variant *ts1*. *Plant Mol Biol* **31**, 631–645.
67. Yeh, S., Moffatt, B.A., Griffith, M. *et al*. (2000) Chitinase genes responsive to cold encode antifreeze proteins in winter cereal. *Plant Physiol* **124**, 1251–1263.
68. Dénarié, J. & Debellé, F. (1996) Rhizobium lipo-chitooligosaccharide nodulation factor: signaling molecules mediating recognition and morphogenesis. *Ann Rev Biochem* **65**, 503–535.
69. Goormachting, S., Lievens, S., Van de Velde, W., Van Montagu, M. & Holsters, M. (1998) Srchi13, a novel early nodulin from *Sesbania rostrata*, is related to acidic class III chitinases. *Plant Cell* **10**, 905–915.
70. Staehelin, C., Granado, J., Müller, J. *et al*. (1994) Perception of Rhizobium nodulation factors by tomato cells and inactivation by root chitinases. *Proc Natl Acad Sci USA* **91**, 2196–2200.
71. Ovtsyna, A.O., Schultze, M., Tikhonovich, I.A. *et al*. (2000) Nod factors of *Rhizobium leguminosarum bv.viciae* and their fucosylated derivatives stimulate a nod factor cleaving activity in pea roots and are hydrolysed *in vitro* by plant chitinases at different rates. *Mol Plant Microbe Interac* **13**, 799–807.
72. Díaz-Perales, A., Collada, C., Blanco, C. *et al*. (1998) Class I chitinases with hevein-like domain, but not class II enzymes, are relevant chestnut and avocado allergens. *J Allergy Clin Immunol* **102**, 127–133.
73. Sowka, S., Hsieh, L.S., Krebitz, M. *et al*. (1998) Identification and cloning of Prs a 1, a 32 kDa endochitinase and major allergen of avocado, and its expression in the yeast *Pichia pastoris*. *J Biol Chem* **273**, 28091–28097.
74. Posch, A., Wheeler, C.H., Chen, Z. *et al*. (1999) Class I endochitinase containing a hevein domain is the causative allergen in latex associated avocado allergy. *Clin Exp Allergy* **29**, 667–672.
75. Blanco, C., Diaz-Perales, A., Collada, C. *et al*. (1999) Class I chitinases as potential panallergens involved in the latex–fruit syndrome. *J Allergy Clin Immunol* **103**, 507–513.
76. Mikkola, J.H., Alenius, H., Kalkkinen, N., Turjanmaa, K., Palosuo, T. & Reunala, T. (1998) Hevein-like protein domains as a possible cause for allergen cross-reactivity between latex and banana. *J Allergy Clin Immunol* **102**, 1005–1012.
77. Sánchez-Monge, R., Blanco, C., Diaz-Perales, A. *et al*. (1999) Isolation and characterization of major banana allergens: identification as fruit class I chitinases. *Clin Exp Allergy* **29**, 673–680.
78. Chen, Z., Posch, A., Cremmer, R., Raulf-Heimsoth, M. & Baur, X. (1998) Identification of hevein (Heb b 6.02) in *Hevea* latex as a major cross-reacting allergen with avocado fruit in patients with latex allergy. *J Allergy Clin Immunol* **102**, 476–481.
79. O'Riordain, Radauer, C., Wagner, S. *et al*. (2001) Hev b 11, a class I chitinase from *Hevea brasiliensis*, and its cross reactivity with Hev b 6.02 hevein. *J Allergy Clin Immunol* **107**, S57.
80. Diaz-Perales, A., Sánchez-Monge, R., Blanco, C., Lombardero, M., Carrillo, T. & Salcedo, G. (2002) What is the role of the hevein-like domain of fruit class I chitinases in their allergenic capacity. *Clin Exp Allergy* **32**, 448–454.
81. Allona, I., Collada, C., Casado, R., Paz-Ares, J. & Aragoncillo, C. (1996) Bacterial expression of an active class Ib chitinase from *Castanea sativa* cotyledons. *Plant Mol Biol* **32**, 1171–1176.
82. Diaz-Perales, A., Collada, C., Blanco, C. *et al*. (1999) Cross-reactions in the latex–fruit syndrome: a relevant role of chitinases but not of complex asparagine-linked glycans. *J Allergy Clin Immunol* **104**, 681–687.
83. Brehler, R., Abrams, E. & Sedlmay, S. (1998) Cross-reactivity between *Ficus benjamina* (weeping fig) and natural rubber-latex. *Allergy* **53**, 402–406.

84. Chen, Z., Düser, M., Flagge, A. *et al*. (2000) Identification and characterization of cross-reactive natural rubber latex and *Ficus benjamina* allergens. *Int Arch Allergy Immunol* **123**, 291–298.
85. Sánchez-Monge, R., Blanco, C., Díaz-Perales, A. *et al*. (2000) Class I chitinases, the panallergens responsible for the latex–fruit syndrome, are induced by ethylene treatment and inactivated by heating. *J Allergy Clin Immunol* **106**, 190–195.
86. Astwood, J.D., Leach, J.N. & Fuchs, R. (1996) Stability of food allergens to digestion *in vitro*. *Nat Biothehcnol* **14**, 1269–1273.
87. Yagami, T., Haishima, Y., Nakamura, A., Osuna, H. & Ikezawa, Z. (2000) Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J Allergy Clin Immunol* **106**, 752–762.
88. Raikhel, N.V., Lee, H.I. & Broekaert, W.F. (1993) Structure and funtion of chitin-binding proteins. *Ann Rev Plant Physiol Plant Mol Biol* **44**, 591–615.
89. Van Parijs, J., Joost, H.M., Peumans, W.J., Geuns, J.M. & Van Laere, A.J. (1992) Effect of *Urtica dioica* agglutinin on germination and cell wall formation of *Phycomyces blakesleeanus*. *Arch Microbiol* **158**, 19–25.
90. Hopkins, T.L. & Harper, M.S. (2001) Lepidopteran peritrophic membranes and effects of dietary wheat germ agglutinin on their formation and structure. *Arch Insect Biochem Physiol* **47**, 100–109.
91. Koo, J.C., Lee, S.Y., Chun, H.J. *et al*. (1998) Two hevein homologs isolated from the seed of *Pharbitis nil* L. exhibit potent antifungal activity. *Biochim Biophys Acta* **1382**, 80–90.
92. Caaveiro, J.M., Molina, A., Gonzalez-Mañas, J.M., Rodriguez-Palenzuela, P., García-Olmedo, F. & Goñi, F.M. (1997) Differential effects of five types of antipathogenic plant peptides on model membranes. *FEBS Lett* **410**, 338–342.
93. Caaveiro, J.M., Molina, A., Rodriguez-Palenzuela, P., Goñi, F.M. & Gonzalez-Mañas, J.M. (1998) Interaction of wheat α-thionin with large unilamellar vesicles. *Protein Sci* **7**, 2567–2577.
94. Hänninen, A.R., Mikkola, J.H., Kalkkinen, N. *et al*. (1999) Increased allergen production in turnip (*Brassica rapa*) by treatments activating defense mechanisms. *J Allergy Clin Immunol* **104**, 194–201.
95. Hänninen, A.R., Kalkkinen, N., Mikkola, J.H. *et al*. (2000) Prohevein-like defense protein of tobacco is a cross-reactive allergen for latex allergic patients. *J Allergy Clin Immunol* **106**, 778–779.

# 7 Profilins

CHRISTIAN RADAUER and
KARIN HOFFMANN-SOMMERGRUBER

## 7.1 Introduction

Profilins are small (12–15 kDa), cytosolic proteins, which are found in all eukaryotic cells. They were first described in 1977 [1] as actin-binding proteins which sequester actin monomers and inhibit actin filament growth *in vitro*. Allergologists' attention was drawn to profilins in 1991, when a highly cross-reactive minor birch pollen allergen, Bet v 2, was identified as a profilin [2]. Since then, profilin cDNAs from numerous plants were cloned and their immunological properties described. It turned out that allergenic profilins are found in nearly every type of plant allergen source such as pollens, fruits, vegetables, spices, seeds, nuts and latex.

Allergic patients whose sera contain profilin-specific IgE commonly show allergic symptoms towards a large number of botanically unrelated plants. However, the extensive *in vitro* cross-reactivity of profilin-specific IgE does not always reflect the clinical sensitisation pattern. The link between *in vitro* sensitisation and apparent clinical symptoms is not fully understood up to now, which poses a problem for the interpretation of diagnostic data, such as measurement of specific IgE. The clarification of this issue requires the integration of molecular biological, biochemical, structural and immunological data. This review gives an overview of the present knowledge of these issues with an emphasis on plant food profilins. After the treatment of general properties of profilins and their role in several cross-reactivity syndromes, the chapter is concluded by a discussion of experimental data and current hypotheses concerning the clinical relevance of profilin-specific IgE in food allergy.

## 7.2 Biochemical and biophysical properties

### 7.2.1 *Sequence data*

A search within the sequences in the Swiss-Prot and translated EMBL databases yielded 67 plant profilin sequences from 31 organisms. Twenty-eight of these sequences are derived from mRNAs isolated from pollen. Similarities among plant profilin sequences are quite high with percentages of identical residues ranging from 70 to 85%. Conversely, homologies to profilins from lower eukaryotes, fungi and animals are much lower: sequence identities range from 25 to 40%.

Plant profilins have a conserved sequence length of 131 to 134 amino acids, which results in molecular masses between 14.0 and 14.5 kDa. The only exceptions are two wheat profilins which are 138 and 140 amino acids in length. The amino acid composition is biased towards acidic residues leading to theoretical isoelectric points between 4.6 and 5.4.

### 7.2.2 *Structure*

Despite low sequence similarity between profilins from mammals, plants and lower eukaryotes, all these proteins share an identical fold. Profilins exhibit a compact globular structure consisting of a central seven-stranded antiparallel beta sheet enclosed by the *N*- and *C*-terminal alpha helices on one side and one or two helices on the other side. Structures of three plant profilins have been determined by X-ray crystallography (Table 7.1): *Arabidopsis thaliana* pollen profilin [3], birch (*Betula verrucosa*) pollen profilin (Bet v 2, [4]) and *Hevea brasiliensis* latex profilin (Hev b 8, Fedorov *et al.*, unpublished results). A comparison of their structures is depicted in Fig. 7.1, a sequence alignment including secondary structure information is shown in Fig. 7.2.

Plant profilins exhibit some structural features that are different from homologues from other organism groups. The segment opposite to the *N*- and *C*-terminal alpha helices contains one helix and a long loop instead of two consecutive short helices. The loop between the *N*-terminal alpha helix and the first beta strand is 3–6 residues longer compared to other profilins. This highly solvent exposed loop comprises the most variable part of the sequence and is part of an IgE epitope in Bet v 2 [4].

The Bet v 2 structure contains a feature not shared by the profilins from *Arabidopsis* and *Hevea* as well as mammals and lower eukaryotes. In all other profilin structures published so far the *N*- and *C*-terminal alpha helices, which are in close proximity in the molecule, are nearly parallel, but the orientation of the *N*-terminal helix with respect to the *C*-terminal helix and the central beta sheet is altered in Bet v 2, leading to an angle of 110° between the two helices. This shift changes the binding site for proline-rich peptides formed by these helices (see below).

7.2.2.1 *Oligomerisation.* The first report describing the oligomerisation of profilin was published in 1996 [5]. Immunoblots of purified human profilin showed that profilin-specific antibodies and G actin bound to a tetramer. The actin-binding capacity of the tetramer was even higher than of the monomer. Analysis by capillary electrophoresis showed that oligomerisation was induced by reducing agents or high salt concentrations. In contrast to these results, a later publication claimed that oligomerisation is induced by oxidising conditions via disulphide bridge formation [6]. The secondary structures of reduced (monomeric) and oxidised (oligomeric) profilins were similar but the thermal stability of monomeric profilin was slightly higher than that of the oligomeric form. Experiments with maize profilin revealed that oligomers formed under non-reducing conditions bound to allergic patients' serum IgE even stronger than monomers [7].

**Table 7.1** Plant profilins with resolved structures included in the Brookhaven Protein Data Bank

| PDB Acc. No. | Protein | References |
|---|---|---|
| 1A0K | *Arabidopsis thaliana* profilin 1 | [3] |
| 3NUL | *Arabidopsis thaliana* profilin 1 (selenomethionine modification) | [3] |
| 1CQA | *Betula verrucosa* pollen profilin (Bet v 2) | [4] |
| 1G5U | *Hevea brasiliensis* latex profilin (Hev b 8) | unpublished |

All data were obtained by X-ray crystallography.

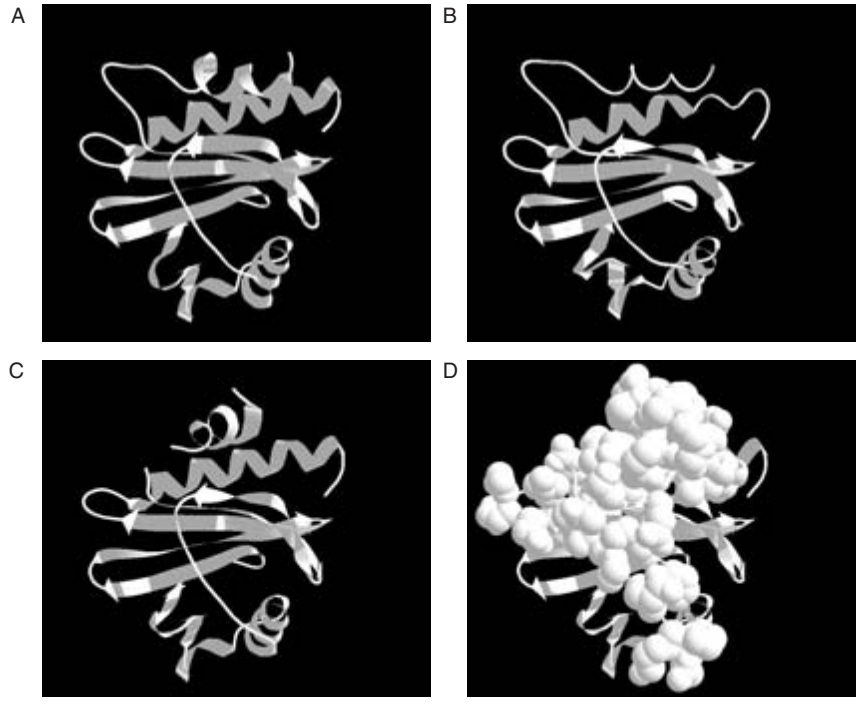


**Fig. 7.1** Structures of plant profilins. **A**: *A. thaliana* pollen profilin (PDB accession number 1A0K); **B**: *H. brasiliensis* latex profilin (1G5U); **C**: birch pollen profilin (1CQA); **D**: linear IgE epitopes of birch pollen profilin (residues 1–20, 36–47 and 108–133) represented as molecular surface. The loop between the *N*-teminal alpha helix and the first beta strand (residues 13–21) had no defined structure in the crystals and is therefore missing. The pictures were generated using the Swiss-PDB Viewer (http://us.expasy.org/spdbv/mainpage.htm, [99]).

### 7.2.3 *Biochemical function*

Profilins fulfil an essential function in all eukaryotic cells, which is illustrated by the fact that disruption of profilin genes is lethal in most organisms tested so far (*Schizosaccharomyces*, *Drosophila*, mouse, reviewed in [8]). Profilins participate in the regulation of polymerisation of actin filaments (also called microfilaments) which are crucial components of the cytoskeleton. The major role of profilin is the rapid reorganisation of microfilaments during processes such as cytokinesis, cell movement, cell elongation and pollen tube growth. In this section, we will focus on the original literature on plant profilins. The much more extensive data on profilins from mammals and lower eukaryotes have been extensively reviewed during recent years [8–10].

7.2.3.1 *Profilin ligands.* Profilin was originally isolated as one of the proteins binding to monomeric actin (G actin) and thus shifting the equilibrium towards actin filament depolymerisation by lowering the free concentration of actin monomers [1]. In contrast, later work showed that profilin promotes the rapid polymerisation of actin filaments even at low concentrations of free G actin by lowering the critical actin

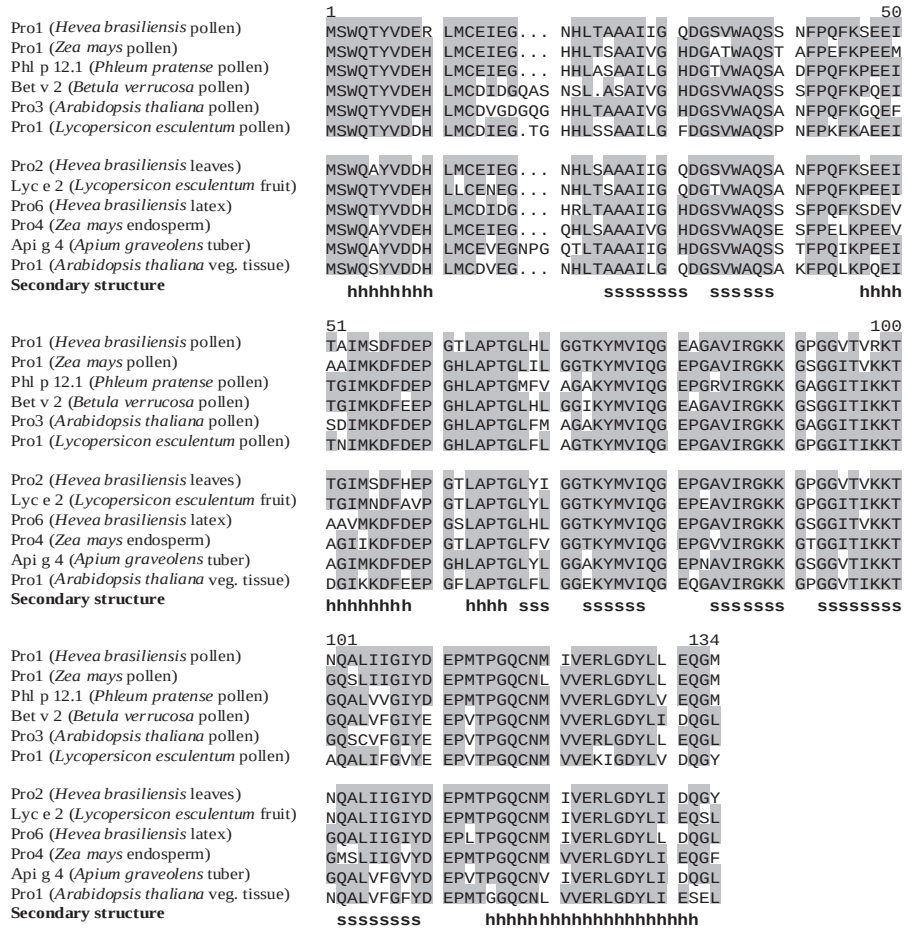


**Fig. 7.2** Alignment of plant profilin sequences expressed in different tissues. Conserved residues are shaded, dots indicate gaps generated in the alignment. A secondary structure assignment deduced from the three experimentally determined plant profilin structures is shown below the sequences (h: alpha helix; s: beta sheet).

concentration in the polymerisation equilibrium (reviewed in [11]). Profilin exerts this function by accelerating the exchange of ADP for ATP on bound actin. The ATP–actin–profilin complex then binds to the plus end of the actin filament which promotes the rapid dissociation of profilin. Actin binds to a surface area formed by parts of the central beta sheet and the *C*-terminal alpha helix. The residues contributing to the binding site are only poorly conserved, but the charge distribution on this surface is quite invariable [3].

Profilins bind phosphatidylinositol phosphates (PIPs) at a site overlapping with the actin-binding site; thus the binding of actin and PIPs is mutually exclusive. Binding of PIPs links the action of profilin on the microfilament system to extracellular signals. Phosphatidylinositol 4,5-bisphosphate (PI-4,5-$P_2$), which is generated as second

messenger in receptor tyrosine kinase-mediated signal transduction, is partially protected from hydrolysis when bound to profilin. In addition, profilin binds to PI-3,4-$P_2$ and PI-3,4,5-$P_3$ with higher affinity than to PI-4,5-$P_2$.

The third group of profilin ligands are proline-rich peptides. The affinity for poly-L-proline (PLP) has been widely used for purification of profilins by affinity chromatography. The biological counterparts of PLP are regulatory proteins containing proline-rich sequences such as the vasodilator-stimulated phosphoprotein (VASP) which plays a role in platelet activation, formin-related proteins which participate in cytokinesis, the establishment of cell polarity and vertebrate limb formation, and adenylyl cyclase-associated protein (CAP). The PLP-binding site, formed by the *N*- and *C*-terminal alpha helices and parts of the beta sheet, is located opposite the actin/PLP-binding patch; thus the two sites do not overlap. Several proline-binding hydrophobic residues are conserved among most profilins. Interestingly, the altered orientation of the *N*-terminal alpha-helix in birch pollen profilin [4] does not affect its PLP-binding capacity though the hydrophobic patch is disrupted. NMR studies with birch pollen profilin [12] showed that the proline-rich sequence must be at least eight amino acids in length. Peptides derived from the sequences of VASP and CAP bind with affinities similar to that of $(Pro)_8$.

7.2.3.2 *The role of profilin in signal transduction.* The data on profilin ligands lead to a model for the link between extracellular signals, profilin and actin filaments (reviewed in [9]). Profilin bound to PI-4,5-$P_2$ is sequestered at the inner surface of the plasma membrane. Phospholipase Cγ1, activated by phosphorylation by receptor tyrosine kinases, cleaves PI-4,5-$P_2$ and thereby releases profilin from the plasma membrane. Free profilin then promotes rapid local actin polymerisation. Binding of formin-related proteins links profilin action to the GTPase-related signalling cascade, whereas binding of proteins from the VASP/Mena family establishes a connection to the adenylate cyclase pathway. Finally, annexin I, a protein whose activity is regulated by the intracellular calcium level, can bind to profilin. Calcium also acts on the actin cytoskeleton by binding to several actin-binding and severing proteins.

### 7.2.4 *Biological function in the plant cell*

The actin cytoskeleton fulfils several important roles in the plant cell (reviewed in [13]):

Actin filaments are involved in many intracellular transport processes such as short-range transport of vesicles and organelles, long-range cytoplasmic streaming, transport of hormones, plastid division and regulation of protein translocation through plasmodesmata. F-actin is also suspected to play a role in translocation of nascent proteins through the ER membrane, as actin filaments have been found associated with polysomes.

The second function of actin filaments is related to cell morphogenesis and division. Reorganisation of the actin cytoskeleton is required for cell elongation, by which most cells in higher plants grow, and tip growth in the specialised cell types, pollen tubes and root hairs. Mitosis is successfully accomplished independently of actin filaments, although they are required for the correct location of the spindle. F-actin is part of the phragmoplast, a structure created during cytokinesis, consisting mainly of microtubules,

which transports exocytotic vesicles to form the cell plate, a precursor of the future cell wall in the division plane. Actin filaments play a role in vesicle transport, the stabilisation of the cell plate, and its final fusion with the existing cell wall.

Finally, changes in cell shape and intracellular transport processes performed by dynamic actin filaments often occur in response to external stimuli. F-actin was found to participate in light-induced chloroplast positioning, opening and closing of stomata in response to light, water stress or $CO_2$ concentration, and gravity sensing in specialised cells, the statocyes. Similar dynamic processes play a role in interactions with pathogens, especially in the course of the hypersensitive response, and wound healing, where cytoplasmic streaming is locally stopped and exocytotic vesicles are targeted at the wounding site by actin filaments.

The role of profilin in many of these processes has been experimentally demonstrated, as shown by a few examples. An important profilin-dependent event is the growth of pollen tubes. Immunofluorescence studies showed elevated profilin concentrations in the growing tips of some pollen tubes of tobacco [14], whereas microscopic examination of germinating lily pollen showed an even distribution of profilin throughout the cytoplasm [15] and microinjection of profilin-inhibited pollen tube growth [16]. The role of profilin in cytokinesis was demonstrated by microinjection into stamen hair cells. When injected during mitosis, profilin delayed or inhibited cell plate formation, reduced cell plate stability and altered its morphology [17]. Injection in interphase cells inhibited cytoplasmic streaming. Experiments with transgenic *Arabidopsis* plants which underexpressed or overexpressed profilin showed that profilin is involved in cell elongation, cell shape maintenance, root hair growth and determination of flowering time [18].

### 7.2.5 *Gene expression and isoforms*

While profilins from animals and lower eukaryotes are encoded by at most three genes, the number of isoforms is larger in plants. The first data on plant profilin isoforms were published in 1993 [19] when three isoforms were isolated from a maize pollen cDNA library. The cloned isoforms proved to be specifically expressed in pollen. The total number of genes was estimated to be between three and six according to Southern blots. Moderate stringency northern blots and affinity purification of profilins from different tissues showed that other isoforms are expressed in all tissues, but the expression level is considerably higher in pollen.

A similar situation is found in *Arabidopsis*, where the number of genes was estimated to be eight [20]. However, a similarity search in the recently completed *Arabidopsis* genome database reveals no profilin sequences in addition to the five ones already described. Comparison of profilin sequences from several monocot and dicot species showed that there are two groups of profilin genes in plants: genes encoding pollen-specific isoforms and isoforms expressed in all tissues. The split between these two groups seems to have occurred a long time ago since sequence similarities between the *Arabidopsis* isoforms of either group and members of the same group from other species (even monocots) are higher than similarities between the two groups [20]. Conversely, a sequence alignment including several foodstuff-derived profilin sequences not known at the time of that publication (Fig. 7.3) yields a more complex

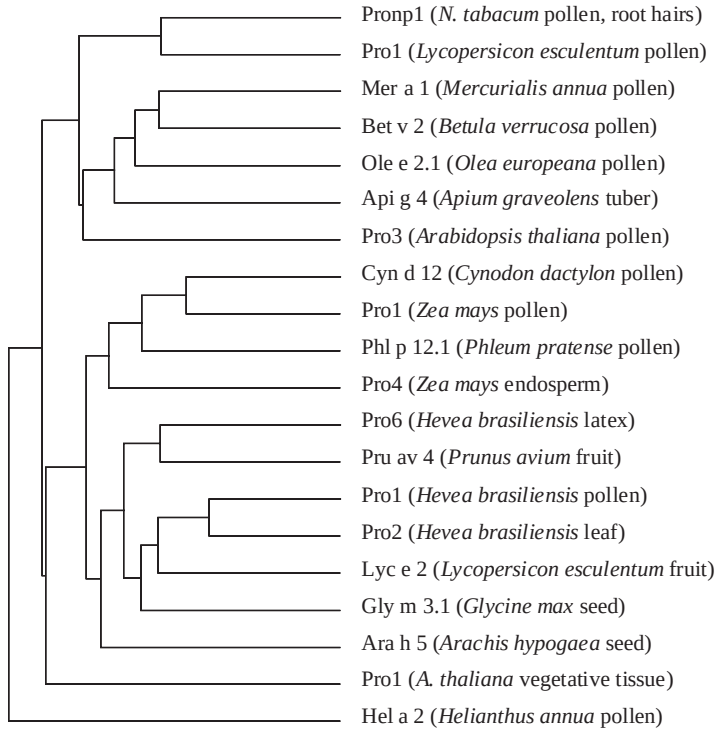


**Fig. 7.3** Phylogenetic tree generated from the multiple sequence alignment shown in Fig. 7.2 including eight additional sequences. The alignment and the tree were generated using the PileUp programme from the GCG package (Accelrys Inc., Madison, WI, USA).

picture: profilin sequences from dicots form two monophyletic groups encompassing mostly pollen-derived and constitutively expressed isoforms, but all types of monocot sequences form a third group equally similar to both dicot clades. In addition, the sequence alignment (Fig. 7.2) reveals no sequence features specific for either group of profilin sequences.

Recently, a novel site of expression for a pollen-specific profilin gene from tobacco, *pronp1*, was discovered [21]. Experiments with the *pronp1* promotor fused to a reporter gene showed that it was highly active not only in mature and germinating pollen but also in root hairs, in contrast to other parts of the root. This reflects the similarity of pollen tube growth and root hair formation with respect to cytoskeleton rearrangement. In the para rubber tree (*Hevea brasiliensis*), cDNAs encoding three quite divergent profilin isoforms were isolated from pollen (Asturias *et al*., EMBL/GenBAnk/DDBJ accession number AJ132397), leaves [22] and latex [23], respectively. The latex isoform probably evolved in response to the highly specialised needs for cytoskeleton regulation in the laticiferous cells.

Profilin gene structures are conserved in plants but differ in yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*) [21]. Plant profilin genes consist of three exons interrupted by two introns of variable length but with conserved insertion sites.

### 7.2.6 *Thermal and gastrointestinal stability*

There are only few data on the stability of profilins, most of them from work on celery profilin. Compared to other allergens, profilin is a moderately stable protein, more resistant than Bet v 1 homologues, but less stable than lipid transfer proteins or cross-reactive carbohydrate determinants of glycoprotein allergens.

Thermal stability was examined in detail using celery extracts [24, 25]. Microwave heating to 100°C for 10 minutes only slightly reduced the IgE-binding capacity of profilin, whereas after 30 minutes almost no IgE-binding activity was detectable in immunoblots and enzyme allergosorbent test (EAST). Conventional cooking in water for 20 minutes did not affect the allergenicity of profilin, and IgE-binding activity was also detected in the cooking water. In accordance with the data obtained from celery, roasting of peanuts did not affect IgE binding of profilin in immunoblots, although the profilin band was split up into a doublet, indicating a certain extent of degradation [26]. In contrast to the results from denaturing immunoblots, native ELISA inhibition of IgE binding to recombinant peanut profilin (Ara h 5) revealed a reduced inhibitory capacity of the roasted peanut extract. This indicated that heating primarily affects conformational IgE epitopes, whose IgE-binding capacity is not detected in immunoblots.

The allergenicity of celery extracts after various food processing treatments was examined using profilin-specific patients' sera [25]. Treatment of celery tubers by gamma-irradiation, drying and powdering, ultra-high pressure treatment, or high voltage impulse treatment did not affect the allergenicity of profilin. However, profilin-specific IgE bound only weakly to extracts of pickled celery treated with vinegar and citric acid. In contrast to commercial celery powder which contained fully active profilin, powdering of bell pepper resulted in the loss of allergenic activity of profilin. While profilin was detected in all eight tested horticultural strains of bell pepper [27], no IgE binding to profilin was observed in paprika powder [28] and binding of profilin-specific IgE to bell pepper extract was not inhibited by paprika powder [27].

Celery extract was also tested for its gastrointestinal stability [29]. Immunoblots showed a slight reduction in the IgE-binding capacity of profilin after gastric digestion, while subsequent pancreatic proteolysis significantly reduced IgE binding. EAST inhibition experiments revealed that the acidic pH of the gastric environment alone was sufficient to destroy conformational IgE epitopes. Fermentation of pea flour by lactic acid bacteria or fungi reduced the allergenicity to 10%, but the profilin contents detected by a rabbit antibody were not affected [30].

One reason for the allergenicity of profilins is their solubility in diluted aqueous solutions. Ten minutes after rehydration of dry birch pollen, extracted profilin was already detected on IgE immunoblots [31].

## 7.3 The role of profilins in allergic cross-reactivity

The role of profilin in allergic reactions to plants was discovered in 1991, when the cDNA encoding birch profilin was isolated from a pollen cDNA library after screening with birch pollen-allergic patients' serum IgE [2]. The allergen, termed Bet v 2, was

shown to be cross-reactive not only with profilins from the closely related trees, alder, hazel, hornbeam and chestnut [32], but also with profilins from grass and weed pollen [33]. Bet v 2-specific IgE even bound weakly to purified human profilin, which also triggered histamine release from basophils isolated from pollen-allergic patients by cross-linking cell-bound profilin-specific IgE [2]. Profilin is now recognised as a minor allergen in nearly every allergenic pollen source examined, and profilin-specific IgE is detected in 10–30% of pollen-allergic patients' sera.

The well-known correlation between allergy to pollen and plant foods suggested a role of profilins in this cross-reactivity, what was corroborated by the detection of IgE-binding profilins in a large number of plant foodstuffs shortly after the cloning of Bet v 2 [34]. Since then, profilin cDNAs have been cloned from nearly every pollen allergen source, and the recombinant proteins have been extensively characterised. Recombinant Bet v 2 is now used in a commercial diagnostic kit for specific IgE measurement. In contrast, only a few profilin cDNAs from plant foods have been isolated (see Table 7.2). Additional data on the allergenicity of plant food profilins have been obtained from studies with purified natural profilins, inhibition studies with recombinant pollen profilins, or analysis of extracts with profilin-specific antibodies and patients' IgE, showing that sensitisation to profilin is found in 10–30% of plant food-allergic patients' sera (see Table 7.3). In the following section, data about the role of profilin in plant food allergy are summarised.

**Table 7.2** Sequence data available from allergenic plant food profilins

| Species | Allergen name | aa | kDa | Nucleotide Acc. No. | Protein Acc. No. | Reference |
|---|---|---|---|---|---|---|
| **Allergens with official designations** | | | | | | |
| Banana (*Musa acuminata*) | Mus xp 1 | 131 | 14.0 | AF377948 | | [74] |
| Bell pepper (*Capsicum annuum*) | Cap a 2 | 131 | 14.2 | AJ417552 | | |
| Celery (*Apium graveolens*) | Api g 4 | 134 | 14.3 | AF129423 | Q9XF37 | [55] |
| Cherry (*Prunus avium*) | Pru av 4 | 131 | 14.0 | AF129425 | Q9XF39 | [43] |
| Hazelnut (*Corylus avellana*) | Cor a 2 | 131 | 14.1 | AF327622 | Q9AXH5 | |
| | | | 14.0 | AF327623 | Q9AXH4 | |
| Lychee (*Litchi sinensis*) | Lit c 1 | 131 | 14.0 | AY049013 | | |
| Peanut (*Arachis hypogaea*) | Ara h 5 | 131 | 14.1 | AF059616 | Q9SQI9 | [76] |
| Pear (*Pyrus communis*) | Pyr c 4 | 131 | 14.1 | AF129424 | Q9XF38 | [43] |
| Pineapple (*Ananas comosus*) | Ana c 1 | 131 | 14.2 | AF377949 | | [74] |
| Soybean (*Glycine max*) | Gly m 3 | 131 | 14.1 | AJ223982 | O65809 | [77] |
| | | | | AJ223981 | O65810 | |
| Tomato (*Lycopersicon esculentum*) | Lyc e 1 | 131 | 14.3 | AJ417553 | | |
| **Allergens without official designations** | | | | | | |
| Apple (*Malus domestica*) | Mal d 4 | 131 | 14.1 | AF129426 | Q9XF40 | |
| | | | 14.1 | AF129427 | Q9XF41 | |
| | | | 14.0 | AF129428 | Q9XF42 | |
| Kidney bean (*Phaseolus vulgaris*) | | 131 | 14.2 | X81982 | P49231 | [97] |
| Wheat (*Triticum aestivum*) | | 138 | 15.0 | X89825 | P49232 | [79] |
| | | 135 | 14.6 | X89826 | P49233 | |
| | | 134 | 14.4 | X89827 | P49234 | |

Allergen names are according to the official allergen list from the WHO/IUIS allergen nomenclature subcommittee, names in parentheses are not yet included in that list.
aa: amino acids; nucleotide accession numbers from EMBL/GenBank/DDBJ; protein accession numbers from Swiss-Prot and TrEMBL.

**Table 7.3** Prevalence of sensitisation to profilin

| Allergen source | Patient group | N | P(%) | Reference |
|---|---|---|---|---|
| Carrot (*Daucus carota*) | Carrot allergics with positive DBPCFC | 20 | 20 | [57] |
| Celery (*Apium graveolens*) | Patients with reported symptoms and positive RAST to celery | 23 | 30 | [50] |
| Celery (*Apium graveolens*) | Patients with at least one pollen and food allergy and EAST > 2 to celery | 60 | 17 | [24] |
| Celery (*Apium graveolens*) | Patients with reported adverse reactions to celery | 30 | 23 | [98] |
| Celery (*Apium graveolens*) | Celery allergics with positive DBPCFC | 22 | 23 | [54] |
| Cherry (*Prunus avium*) | Birch allergics with cherry allergy (positive case history and EAST) | 101 | 16 | [45] |
| Peach (*Prunus persica*) | Patients with positive case history, SPT and RAST to peach (with and without pollen allergy) | 21 | 57 | [44] |
| Hazelnut (*Corylus avellana*) | Patients with allergies to birch pollen and hazelnuts | 25 | 8 | [40] |
| Peanut (*Arachis hypogaea*) | Peanut-allergic patients with positive case history, SPT and RAST | 40 | 13 | [77] |
| Peanut (*Arachis hypogaea*) | Peanut-allergic patients with OAS | 50 | 16 | [26] |

N: number of patients; P: prevalence.

### 7.3.1 *Hazelnuts and Rosaceae fruits*

Up to 70% of birch pollen-allergic patients suffer from allergies to hazelnuts, apples, pears, stone fruits or kiwi [35–37]. In most cases this correlation can be attributed to cross-reactivity between the major birch pollen allergen, Bet v 1 [38], and its homologues in these fruits [39], but profilin cross-reactivity plays a role in 10–20% of the patients.

The first report supporting a role of profilins in this syndrome was published in 1992 [40] showing that the frequently observed cross-reactivity between birch pollen, hazel pollen and hazelnuts is caused in some patients by profilin. Two out of 25 sera from patients allergic to birch pollen and hazelnuts recognised profilin in IgE immunoblots. The cDNA sequences of two isoforms of hazelnut profilin were recently submitted to the EMBL/GenBank/DDBJ databases (see Table 7.2), but no data on the properties of these proteins were published.

The fruit most frequently causing allergic reactions is apple (*Malus domestica*). While most cases of apple allergy in central and northern Europe can be attributed to the cross-reaction between Bet v 1 and its apple homologue, Mal d 1 [41], allergic reactions to apples are commonly caused by profilin in regions where birch trees are rare, such as the Mediterranean area. While only four out of twenty birch pollen and fruit-allergic patients in an Austrian study had profilin-specific IgE detected by immunoblots [39], the majority of grass pollen and fruit-allergic patients from central Spain recognised profilin in skin prick tests or radioallergosorbent test (RAST) [42]. cDNAs encoding three profilin isoforms were recently cloned from apple fruit RNA and the allergen provisionally termed Mal d 4 (Scheurer *et al.*, unpublished results, see Table 7.2).

Although allergy to pear (*Pyrus communis*) is significantly rarer than apple allergy, birch and apple profilin-specific IgE from all patients tested also recognised pear

profilin [39]. The cDNA encoding pear profilin (Pyr c 4) was cloned and the recombinant protein expressed in *E. coli* [43].

Stone fruits of the subfamily *Prunoideae* (peach, apricot, cherry, plum) often cause allergic reactions in birch pollen-allergic patients. In addition to the Bet v 1 homologues, profilins are a major cause of this cross-reactivity. In an Italian study including 21 peach-allergic patients, most of them with an additional pollen allergy to birch and/or grass pollen, between 33% and 57% of the sera recognised the profilins of peach, cherry and plum in IgE immunoblots [44]. In a group of 101 birch pollen-allergic patients with concomitant cherry allergy, only 16% were sensitised to profilin [45], reflecting the lower sensitisation potential of birch pollen profilin compared to grass pollen profilins. Allergy to stone fruits is sometimes not accompanied by pollen allergy, especially in regions without birch trees. These patients frequently suffer from severe symptoms due to sensitisation to the heat-stable cross-reactive allergen, lipid transfer protein (see Chapter 4). They only rarely show IgE directed to profilin [45, 46].

### 7.3.2 *Vegetables and spices from the family Apiaceae*

Mugwort-allergic patients frequently show allergic reactions to vegetables and spices from the family Apiaceae such as celery, carrot, parsley, anise, fennel, coriander, cumin and caraway [47–49]. This correlation, termed the celery–carrot–mugwort–spice syndrome, also includes allergy to birch pollen. The cross-reactivity can be attributed to three cross-reactive allergens [50]: the major birch pollen allergen, Bet v 1, and its celery homologue, Api g 1, a set of high molecular weight allergens most probably bearing cross-reactive carbohydrate determinants and profilins.

Celery (*Apium graveolens*) is the food most frequently eliciting allergic reaction in Central Europe [51]. Celery profilin (Api g 4) was the first food profilin described as an allergen [52]. A rabbit antiserum raised against the purified protein cross-reacted with profilins from birch, ragweed and grass pollen as well as from apple and carrot. The cross-reactivity with birch and mugwort pollen [50] as well as apple [53] profilins was also shown by immunoblot inhibition. Several studies revealed that between 20% and 30% of celery-allergic patients' sera contain profilin-specific IgE (see Table 7.3), including a group of patients with celery allergy confirmed by double-blind placebo-controlled food challenge (DBPCFC [54]). No significant differences were detected between asymptomatic patients with positive celery RAST and real celery allergics [50, 54]. The cDNA encoding Api g 4 was cloned, and the recombinant protein expressed in *E. coli* bound to patients' IgE and induced histamine release from basophils isolated from allergic patients' blood [55].

Celery-allergic patients frequently show allergic symptoms to other members of the Apiaceae family such as carrot, parsley, anise, fennel, coriander, cumin and caraway [47]. Profilin was detected in extracts from anise, coriander, fennel and cumin by an anti-celery profilin antibody [56]. Only pollen-allergic patients cross-sensitised to spices, but not patients monosensitised to spices, showed spice-specific IgE in immunoblots. Profilin was recognised by 20% of the atopic patients in extracts from anise and fennel, but not coriander and cumin. Furthermore, IgE directed to carrot profilin was detected in 20% of sera from patients with carrot allergy confirmed by DBPCFC [57].

IgE binding to Apiaceae profilins was completely inhibited by recombinant Bet v 2, recombinant Api g 4, and extracts from celery, birch and mugwort [56, 57].

### 7.3.3 *Solanaceae vegetables*

Birch and mugwort pollen-allergic patients frequently show adverse reactions to potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*) and bell pepper (*Capsicum annuum*), members of the family *Solanaceae*. Tomato is also one of the foods commonly eliciting allergic reaction in patients suffering from allergy to grass pollen as the only inhalative allergen source [58].

Profilin-specific IgE from birch pollen allergic patients with fruit allergy recognised profilins from potato [39], which is fully cross-reactive to Bet v 2. An anti-celery profilin antibody detected profilin in all eight tested horticultural strains of bell pepper [27]. Five out of eleven pollen-allergic patients with bell pepper allergy had IgE directed to profilin. Conversely, no profilin was detected in paprika powder [28] and IgE binding to bell pepper profilin was not inhibited by paprika extract [27].

In an immunoblot examination of eight sera from tomato-allergic patients, who all suffered from grass pollen allergy, five sera recognised profilin [59], which is, besides cross-reactive carbohydrate determinants, the cause for cross-reactivity between grass pollen and tomatoes. The cDNAs encoding profilins from tomato and bell pepper, Lyc e 1 and Cap a 2, were recently cloned [100] (see Table 7.2). The sequence of the tomato fruit profilin is different from the pollen sequence previously published [60].

### 7.3.4 Hevea brasiliensis *latex profilin and the latex–fruit syndrome*

About half of the *Hevea brasiliensis* latex-allergic patients suffer from allergies to avocado, banana, kiwi, chestnut, or various other, mainly tropical, fruits [61], a condition called the latex–fruit syndrome [62]. The cross-reactivity is mainly attributed to the major latex allergen hevein (Hev b 6.02) and hevein-like domains in class I chitinases [63, 64], latex beta-1,3-glucanase (Hev b 2) and its homologues in fruits [65], and homologues of the chitinase/lysozyme hevamine [66].

The role of profilin in this syndrome is currently a subject of debate. The presence of profilin in latex extracts was first described in 1995 [67, 68]. Two out of nineteen latex-allergic patients recognised profilin on immunoblots of latex extract [68]. Latex profilin was cross-reactive with ragweed profilin and Bet v 2. Only minimal amounts of profilin were found in the latex rubber fraction and in latex glove extracts. Fifty-nine per cent of ragweed-allergic patients without symptoms to latex had a positive latex CAP (Capsulated Hydrophilic Carrier Polymer Immunoassay). Taking profilin sensitisation into account, the percentages were 100% for profilin-positive and only 15% for profilin-negative sera. These data led to the conclusion that sensitisation to latex profilin via pollen or food profilins does not elicit clinical symptoms of latex allergy. Similar results were found in a Spanish examination of patients with pollen and food allergy [69]. Many pollen-allergic patients showed a positive latex CAP without symptoms of latex allergy. Recombinant latex profilin (Hev b 8) cDNAs were cloned from *Hevea brasiliensis* leaves [22] and latex [23]. The frequency of profilin

sensitisation among latex-allergic patients was 20–25% with adult subjects and 6–12% with children suffering from spina bifida, a high-risk group for latex allergy. Latex profilin was fully cross-reactive with profilins from birch, mugwort and grass pollen as well as celery [23]. Interestingly, no latex-monosensitised patient's serum recognising profilin was found among 84 patients tested. Skin prick tests with recombinant Hev b 8 resulted in only one weakly positive reaction in 29 patients tested [70]. This is in contrast with intradermal tests performed by Nieto and coworkers [71], where nearly all tested patients reacted to natural and recombinant Hev b 8.

Almost no data are available about the role of profilin in the latex–fruit syndrome. Several studies suggest a role of profilin in kiwi allergy, a condition related to birch pollen as well as latex allergy: 26% of kiwi-allergic patients recognised a 14 kDa allergen, whose IgE-binding capacity could be abolished by preincubation of the sera with birch pollen extract [72]. A different study determined a frequency of 9% for IgE recognition of a 13 kDa allergen identified as profilin by an anti-profilin antiserum [73]. IgE binding to profilin was also found in banana-allergic patients [68]. Recently, profilins from banana, lychee and pineapple were cloned [74] (see Table 7.2). In addition, several studies of the cross-reactivity between latex and pollen allergens suggest the contribution of profilin to this syndrome [69, 75, 76].

### 7.3.5 *Legumes*

Patients allergic to grass pollen without another inhalative allergy commonly exhibit adverse reaction towards tomatoes, peanuts, green peas and wheat [59]. This cross-reactivity may be caused in part by profilin. The cDNA of peanut (*Arachis hypogaea*) profilin, Ara h 5, was isolated from a phage display cDNA library after screening with patients' sera [77]. Thirteen per cent of a group of peanut-allergic patients showed profilin-specific IgE. Peanut-allergic patients sensitised to profilin, but not to the major peanut allergens, Ara h 1 and Ara h 2, showed only mild symptoms such as the oral allergy syndrome [26]. The prevalence of profilin sensitisation in a group of peanut-allergic patients with oral allergy syndrome was 16%. Interestingly, recombinant Ara h 5 was only partially cross-reactive to profilins from other allergen sources (see Section 7.4).

Two profilin isoforms were cloned from soybean (*Glycine max*) [78]. Nine out of 13 soybean-allergic patients' sera recognised the recombinant protein. Soybean profilin (Gly m 3) completely blocked IgE binding to Bet v 2.

### 7.3.6 *Other allergenic profilins*

Three profilin isoforms were cloned from a wheat seedling cDNA library [79], but no data on the allergenicity of the proteins were obtained. Despite the importance of profilins in grass and cereal pollen allergy, they seem to play no role in food allergy to cereals.

Profilin plays a role in some rather rare allergies to seeds. IgE from three pollen-allergic patients with pumpkin seed allergy recognised a 14 kDa allergen cross-reactive to Bet v 2 [80]. Likewise, five out of eleven poppy seed-allergic patients had profilin-specific IgE [81]. Although one third of patients allergic to sunflower

pollen recognise profilin, nothing is known about the relevant allergens in sunflower seeds [82].

Finally, IgE reactivity to profilin was detected in sera of patients allergic to various fruits and vegetables such as lychee [83], zucchini [84] and persimmon [85].

## 7.4 Immunological properties

### 7.4.1 *Cross-reactivity of profilin-specific IgE*

Most studies confirm the more or less full immunological equivalence of profilins from different sources, meaning that profilin-sensitised patients' IgE recognise all tested profilins with similar affinity, and IgE binding to one profilin is inhibited by other profilins from botanically unrelated plants. Examples of this are: IgE from profilin-sensitised patients with birch–fruit syndrome bound to profilins from apple, pear, celery, carrot and potato. Moreover, IgE binding to these fruits was completely inhibited by Bet v 2 [39]. IgE binding to profilins from Apiaceae spices and carrot was completely abolished by Bet v 2 and extracts from mugwort, birch and celery [56, 57]. Likewise, latex profilin fully inhibited IgE binding to profilins from birch, grass and celery. This inhibition also worked in the opposite direction [23].

On the other hand, there are several reports suggesting the existence of species-specific IgE epitopes on profilin, even among members of the same botanical family. Binding of latex-allergic patients' IgE to purified latex profilin was not inhibited by *Mercurialis annua* pollen profilin, a weed belonging, like *Hevea brasiliensis*, to the *Euphorbiaceae* family [86]. A study including 49 birch pollen-allergic patients sensitised to Bet v 2 showed that only between 39 and 45 sera recognised profilins from celery, pear or cherry [43]. Inhibition studies with sera recognising all tested food profilins resulted in only partial inhibition of binding to Bet v 2 by the food profilins. These results indicated that the order of sensitisation affects the IgE-binding pattern. Patients are sensitised to Bet v 2 and recognise cross-reactive and Bet v 2-specific, but not food profilin-specific, epitopes. Similar results were obtained with sera from peanut-allergic patients. Binding to peanut profilin (Ara h 5) was completely abolished by Pru av 4, but only partially inhibited by Api g 4 and Pyr c 4, whereas no inhibition was achieved with Bet v 2 [26]. This probably also reflects sequence differences between pollen and constitutive isoforms.

### 7.4.2 *IgE epitopes*

IgE binds to both linear and conformational epitopes on profilin. Linear epitopes of birch pollen profilin were characterised by screening a library generated from a randomly fragmented Bet v 2 cDNA [4]. Most IgE-binding fragments were clustered in three regions: the *N*-terminal alpha helix and the succeeding long, solvent-exposed loop, a long loop between the central beta sheet and the helix opposite to the *N*- and *C*-terminal helices, and the *C*-terminal alpha helix (see Fig. 7.1). These regions comprise all possible antibody-binding sites since all other loops are too short to harbour a complete epitope and the alternating side chain conformation in the central beta sheet prohibits its substitution by a short peptide.

The participation of conformational epitopes in IgE-binding to profilins can be deduced from several studies: three long (50–80 amino acids) overlapping fragments of soybean profilin (Gly m 3) bound only weakly to IgE and only partially inhibited IgE-binding to intact Gly m 3 [78]. Only 11 out of 14 sera reactive to native purified olive pollen profilin in ELISA bound to the partially denatured protein on immunoblots [87]. The sequence of a peptide mimotope mimicking a cross-reactive IgE epitope of birch, mugwort and celery profilins revealed no similarity with the linear sequences of these proteins [88]. However, the mimotope was capable of inhibiting IgE binding to these profilins by 0–82%, depending on the protein and the serum. A similar patient-dependent distribution of epitopes was found in the experiments with soybean profilin cited above [77].

### 7.4.3 *T-cell reactivity*

There are only few studies dealing with the T-cell reactivity of profilins. T cells from grass pollen-allergic patients were stimulated with purified timothy grass profilin (Phl p 12). The stimulation indices were elevated compared to T cells from non-allergic control donors. Eight Phl p 12-specific T-cell lines did not cross-react with *Parietaria judaica* pollen profilin [89]. An evaluation of the T-cell response of 14 birch pollen-allergic patients showed that T-cell reactivity to Bet v 2 was low in all patients, compared to birch pollen extract and Bet v 1 [90]. Another study examined late cutaneous reaction to food challenge in birch pollen-allergic atopic dermatitis patients [91]. A comparison of T-cell responses in patients responding to food challenge with a deterioration of their eczematous skin lesions with T-cell reactivity of non-responders showed significantly higher simulation indices for birch pollen extract and Bet v 1, but not for Bet v 2.

## 7.5 The clinical significance of profilin-specific immunoglobulin E

A major problem of allergy diagnosis, especially when concerning food allergy, is the reliability of standard tests such as skin tests or, above all, specific IgE measurements. The problem is exacerbated by highly cross-reactive allergens like profilin, whose wide cross-reactivity *in vitro* is not always reflected by comparable clinical manifestations. In the following section, data concerning the clinical relevance of IgE directed to profilin are summarised.

Several studies clearly demonstrated a correlation between sensitisation to profilin and allergic symptoms to multiple pollen sources. In a large study including more than one thousand patients, 55% of patients with positive skin prick tests to multiple pollen species had a positive Bet v 2 CAP, but none of the mono-or oligosensitised patients [92]. Similar results were obtained from a smaller Italian study examining cross-reactivity between pollens and food. All Bet v 2-positive patients had positive SPT to birch, grass and olive pollens [93]. Likewise, all Bet v 2 SPT-positive birch pollen-allergic patients included in a French study displayed a positive SPT and symptoms to grass or weed pollen [94]. On the other hand, in this study three patients were found who were monosensitised to profilin and showed a positive birch pollen

SPT without allergic symptoms during the birch season but a typical grass or weed pollinosis instead.

The situation concerning the association of profilin sensitisation and food allergy is less clear. A comparison of reported symptoms of food allergy with results of CAP assays to pollen-related food in 274 pollen-allergic patients showed that food-specific IgE is found in many of these patients, even without clinical manifestation of food allergy [95]. The opposite case, positive case history but negative CAP, was only rarely observed. Conversely, IgE directed to profilin seems to be a slightly more specific marker for pollen-related food allergy than measurement of specific IgE using whole extracts. Several studies showed that profilin-sensitised patients have an increased probability of food allergy, although the risk for Bet v 1-sensitised patients is even higher [93, 96]. Nevertheless, many profilin-sensitised patients have no food allergy. In a study including 49 birch pollen-allergic patients sensitised to Bet v 2, allergic symptoms to cherry, pear and celery were compared with specific IgE to the respective food profilins [43]. Each foodstuff caused allergic reactions in about half of the patients. Nearly all food-allergic patients, but also most non-allergic patients, recognised the particular food profilins. The authors concluded that specific IgE to profilin is a very sensitive, but quite non-specific marker for pollen-related food allergy. A comparison of birch pollen-allergic patients with and without hazelnut allergy yielded a different result [40]. All patients, irrespective of a hazelnut allergy, had IgE directed to Bet v 1 and its hazel pollen homologue, Cor a 1, and most of them also recognised a similar protein in hazelnut extracts. On the other hand, all profilin-sensitised patients had a hazelnut allergy.

Examination of the IgE recognition patterns of peanut-allergic patients showed that profilin-sensitised patients, most probably sensitised by grass pollen, suffered only from mild symptoms (oral allergy syndrome) after ingestion of peanuts, whereas patients with IgE directed to the more stable peanut-specific allergens, such as Ara h 1 and Ara h 2, often displayed severe symptoms including anaphylactic shock [26]. Another example of a correlation between profilin sensitisation and particular symptoms was shown for patients with multiple pollen sensitisation. Profilin-sensitised patients showed a reduced probability of severe asthma compared to patients sensitised to another pollen pan-allergen, June 2 [92].

In order to summarise the results, the following conclusions can be drawn: (i) profilin-sensitised patients have an increased risk of developing allergy to multiple pollen species, (ii) profilin sensitisation likewise raises the probability of pollen-related food allergies though many profilin-sensitised patients never develop such a condition, and (iii) profilin-mediated allergy is usually confined to mild, local symptoms, like rhinoconjunctivitis and oral allergy syndrome.

## References

1. Carlsson, L., Nystrom, L.E., Sundkvist, I., Markey, F. & Lindberg, U. (1977) Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J Mol Biol* **115**, 465–483.
2. Valenta, R., Duchene, M., Pettenburger, K. *et al.* (1991) Identification of profilin as a novel pollen allergen: IgE autoreactivity in sensitized individuals. *Science* **253**, 557–560.

3. Thorn, K.S., Christensen, H.E., Shigeta, R. *et al*. (1997) The crystal structure of a major allergen from plants. *Structure* **5**, 19–32.
4. Fedorov, A.A., Ball, T., Mahoney, N.M., Valenta, R. & Almo, S.C. (1997) The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. *Structure* **5**, 33–45.
5. Babich, M., Foti, L.R., Sykaluk, L.L. & Clark, C.R. (1996) Profilin forms tetramers that bind to G-actin. *Biochem Biophys Res Commun* **218**, 125–131.
6. Mittermann, I., Fetrow, J.S., Schaak, D.L. *et al*. (1998) Oligomerization of profilins from birch, man and yeast. Profilin, a ligand for itself? *Sex Plant Reprod* **11**, 183–191.
7. Psaradellis, T., Kao, N.S. & Babich, M. (2000) Recombinant Zea mays profilin forms multimers with pan-allergenic potential. *Allergol Int* **49**, 27–35.
8. Ayscough, K.R. (1998) In vivo functions of actin-binding proteins. *Curr Opin Cell Biol* **10**, 102–111.
9. Schluter, K., Jockusch, B.M. & Rothkegel, M. (1997) Profilins as regulators of actin dynamics. *Biochim Biophys Acta* **1359**, 97–109.
10. Wear, M.A., Schafer, D.A. & Cooper, J.A. (2000) Actin dynamics: assembly and disassembly of actin networks. *Curr Biol* **10**, R891–R895.
11. Weber, A. (1999) Actin binding proteins that change extent and rate of actin monomer-polymer distribution by different mechanisms. *Mol Cell Biochem* **190**, 67–74.
12. Domke, T., Federau, T., Schluter, K. *et al*. (1997) Birch pollen profilin: structural organization and interaction with poly-(L-proline) peptides as revealed by NMR. *FEBS Lett* **411**, 291–295.
13. Volkmann, D. & Baluska, F. (1999) Actin cytoskeleton in plants: from transport networks to signaling networks. *Microsc Res Tech* **47**, 135–154.
14. Mittermann, I., Swoboda, I., Pierson, E. *et al*. (1995) Molecular cloning and characterization of profilin from tobacco (*Nicotiana tabacum*): increased profilin expression during pollen maturation. *Plant Mol Biol* **27**, 137–146.
15. Vidali, L. & Hepler, P.K. (1997) Characterization and localization of profilin in pollen grains and tubes of Lilium longiflorum. *Cell Motil Cytoskeleton* **36**, 323–338.
16. Vidali, L., McKenna, S.T. & Hepler, P.K. (2001) Actin polymerization is essential for pollen tube growth. *Mol Biol Cell* **12**, 2534–2545.
17. Valster, A.H., Pierson, E.S., Valenta, R., Hepler, P.K. & Emons, A.M.C. (1997) Probing the plant actin cytoskeleton during cytokinesis and interphase by profilin microinjection. *Plant Cell* **9**, 1815–1824.
18. Ramachandran, S., Christensen, H.E., Ishimaru, Y. *et al*. (2000) Profilin plays a role in cell elongation, cell shape maintenance, and flowering in *Arabidopsis*. *Plant Physiol* **124**, 1637–1647.
19. Staiger, C.J., Goodbody, K.C., Hussey, P.J., Valenta, R., Drobak, B.K. & Lloyd, C.W. (1993) The profilin multigene family of maize: differential expression of three isoforms. *Plant J* **4**, 631–641.
20. Huang, S., McDowell, J.M., Weise, M.J. & Meagher, R.B. (1996) The *Arabidopsis* profilin gene family. Evidence for an ancient split between constitutive and pollen-specific profilin genes. *Plant Physiol* **111**, 115–126.
21. Swoboda, I., Bhalla, P.L., Xu, H. *et al*. (2001) Identification of pronp1, a tobacco profilin gene activated in tip-growing cells. *Plant Mol Biol* **46**, 531–538.
22. Rihs, H.P., Chen, Z., Rozynek, P., Baur, X., Lundberg, M. & Cremer, R. (2000) PCR-based cloning, isolation, and IgE-binding properties of recombinant latex profilin (rHev b 8). *Allergy* **55**, 712–717.
23. Ganglberger, E., Radauer, C., Wagner, S. *et al*. (2001) Hev b 8, the *Hevea brasiliensis* latex profilin, is a cross-reactive allergen of latex, plant foods and pollen. *Int Arch Allergy Immunol* **125**, 216–227.
24. Jankiewicz, A., Aulepp, H., Baltes, W. *et al*. (1996) Allergic sensitization to native and heated celery root in pollen-sensitive patients investigated by skin test and IgE binding. *Int Arch Allergy Immunol* **111**, 268–278.
25. Jankiewicz, A., Baltes, W., Bögl, K.W. *et al*. (1997) Influence of food processing on the immunochemical stability of celery allergens. *J Sci Food Agric* **75**, 359–370.
26. Kleber-Janke, T., Crameri, R., Scheurer, S., Vieths, S. & Becker, W.M. (2001) Patient-tailored cloning of allergens by phage display: peanut (*Arachis hypogaea*) profilin, a food allergen derived from a rare mRNA. *J Chromatogr B Biomed Sci Appl* **756**, 295–305.
27. Jensen-Jarolim, E., Santner, B., Leitner, A. *et al*. (1998) Bell peppers (*Capsicum annuum*) express allergens (profilin, pathogenesis-related protein P23 and Bet v 1) depending on the horticultural strain. *Int Arch Allergy Immunol* **116**, 103–109.
28. Leitner, A., Jensen-Jarolim, E., Grimm, R. *et al*. (1998) Allergens in pepper and paprika. Immunologic investigation of the celery–birch–mugwort–spice syndrome. *Allergy* **53**, 36–41.
29. Jankiewicz, A., Baltes, W., Bögl, K.W. *et al*. (1997) *In vitro* study of the gastointestinal stability of celery allergens. *Food Agricult Immunol* **9**, 203–217.
30. Barkholt, V., Jorgensen, P.B., Sorensen, D. *et al*. (1998) Protein modification by fermentation: effect of fermentation on the potential allergenicity of pea. *Allergy* **53**, 106–108.

31. Vrtala, S., Grote, M., Duchene, M. *et al*. (1993) Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int Arch Allergy Immunol* **102**, 160–169.
32. Hirschwehr, R., Jager, S., Horak, F. *et al*. (1993) Allergens from birch pollen and pollen of the European chestnut share common epitopes. *Clin Exp Allergy* **23**, 755–761.
33. Valenta, R., Duchene, M., Ebner, C. *et al*. (1992) Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* **175**, 377–385.
34. van Ree, R., Voitenko, V., van Leeuwen, W.A. & Aalberse, R.C. (1992) Profilin is a cross-reactive allergen in pollen and vegetable foods. *Int Arch Allergy Immunol* **98**, 97–104.
35. Halmepuro, L., Vuontela, K., Kalimo, K. & Bjorksten, F. (1984) Cross-reactivity of IgE antibodies with allergens in birch pollen, fruits and vegetables. *Int Arch Allergy Appl Immunol* **74**, 235–240.
36. Calkhoven, P.G., Aalbers, M., Koshte, V.L., Pos, O., Oei, H.D. & Aalberse, R.C. (1987) Cross-reactivity among birch pollen, vegetables and fruits as detected by IgE antibodies is due to at least three distinct cross-reactive structures. *Allergy* **42**, 382–390.
37. Gall, H., Kalveram, K.J., Forck, G. & Sterry, W. (1994) Kiwi fruit allergy: a new birch pollen-associated food allergy. *J Allergy Clin Immunol* **94**, 70–76.
38. Breiteneder, H., Pettenburger, K., Bito, A. *et al*. (1989) The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene. *Embo J* **8**, 1935–1938.
39. Ebner, C., Hirschwehr, R., Bauer, L. *et al*. (1995) Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* **95**, 962–969.
40. Hirschwehr, R., Valenta, R., Ebner, C. *et al*. (1992) Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J Allergy Clin Immunol* **90**, 927–936.
41. Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Laimer da Camara Machado, M. *et al*. (1995) Cloning and sequencing of Mal d 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to Bet v 1, the major birch pollen allergen. *Biochem Biophys Res Commun* **214**, 538–551.
42. van Ree, R., Fernandez-Rivas, M., Cuevas, M., van Wijngaarden, M. & Aalberse, R.C. (1995) Pollen-related allergy to peach and apple: an important role for profilin. *J Allergy Clin Immunol* **95**, 726–734.
43. Scheurer, S., Wangorsch, A., Nerkamp, J. *et al*. (2001) Cross-reactivity within the profilin panallergen family investigated by comparison of recombinant profilins from pear (Pyr c 4), cherry (Pru av 4) and celery (Api g 4) with birch pollen profilin Bet v 2. *J Chromatogr B Biomed Sci Appl* **756**, 315–325.
44. Pastorello, E.A., Ortolani, C., Farioli, L. *et al*. (1994) Allergenic cross-reactivity among peach, apricot, plum, and cherry in patients with oral allergy syndrome: an *in vivo* and *in vitro* study. *J Allergy Clin Immunol* **94**, 699–707.
45. Scheurer, S., Pastorello, E.A., Wangorsch, A., Kastner, M., Haustein, D. & Vieths, S. (2001) Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the *in vitro* diagnosis of cherry allergy. *J Allergy Clin Immunol* **107**, 724–731.
46. Fernandez-Rivas, M., van Ree, R. & Cuevas, M. (1997) Allergy to Rosaceae fruits without related pollinosis. *J Allergy Clin Immunol* **100**, 728–733.
47. Wuthrich, B. & Dietschi, R. (1985) The celery–carrot–mugwort–condiment syndrome: skin test and RAST results. *Schweiz Med Wochenschr* **115**, 258–264.
48. Pauli, G., Bessot, J.C., Dietemann-Molard, A., Braun, P.A. & Thierry, R. (1985) Celery sensitivity: clinical and immunological correlations with pollen allergy. *Clin Allergy* **15**, 273–279.
49. Vallier, P., Dechamp, C., Vial, O. & Deviller, P. (1988) A study of allergens in celery with cross-sensitivity to mugwort and birch pollens. *Clin Allergy* **18**, 491–500.
50. Bauer, L., Ebner, C., Hirschwehr, R. *et al*. (1996) IgE cross-reactivity between birch pollen, mugwort pollen and celery is due to at least three distinct cross-reacting allergens: immunoblot investigation of the birch–mugwort–celery syndrome. *Clin Exp Allergy* **26**, 1161–1170.
51. Wüthrich, B. (1993) Food allergy. *Allergologie* **16**, 280–287.
52. Vallier, P., DeChamp, C., Valenta, R., Vial, O. & Deviller, P. (1992) Purification and characterization of an allergen from celery immunochemically related to an allergen present in several other plant species. Identification as a profilin. *Clin Exp Allergy* **22**, 774–782.
53. Rudeschko, O., Fahlbusch, B., Henzgen, M., Schlenvoigt, G. & Jäger, L. (1996) Cross-reactivity of celery and apple allergens. *Allergologie* **19**, 361–366.
54. Luttkopf, D., Ballmer-Weber, B.K., Wuthrich, B. & Vieths, S. (2000) Celery allergens in patients with positive double-blind placebo-controlled food challenge. *J Allergy Clin Immunol* **106**, 390–399.
55. Scheurer, S., Wangorsch, A., Haustein, D. & Vieths, S. (2000) Cloning of the minor allergen Api g 4 profilin from celery (*Apium graveolens*) and its cross-reactivity with birch pollen profilin Bet v 2. *Clin Exp Allergy* **30**, 962–971.

56. Jensen-Jarolim, E., Leitner, A., Hirschwehr, R. *et al.* (1997) Characterization of allergens in Apiaceae spices: anise, fennel, coriander and cumin. *Clin Exp Allergy* **27**, 1299–1306.
57. Ballmer-Weber, B.K., Wuthrich, B., Wangorsch, A., Fotisch, K., Altmann, F. & Vieths, S. (2001) Carrot allergy: double-blinded, placebo-controlled food challenge and identification of allergens. *J Allergy Clin Immunol* **108**, 301–307.
58. de Martino, M., Novembre, E., Cozza, G., de Marco, A., Bonazza, P. & Vierucci, A. (1988) Sensitivity to tomato and peanut allergens in children monosensitized to grass pollen. *Allergy* **43**, 206–213.
59. Petersen, A., Vieths, S., Aulepp, H., Schlaak, M. & Becker, W.M. (1996) Ubiquitous structures responsible for IgE cross-reactivity between tomato fruit and grass pollen allergens. *J Allergy Clin Immunol* **98**, 805–815.
60. Yu, L.X., Nasrallah, J., Valenta, R. & Parthasarathy, M.V. (1998) Molecular cloning and mRNA localization of tomato pollen profilin. *Plant Mol Biol* **36**, 699–707.
61. Lavaud, F., Sabouraud, D., Deschamps, F. & Perdu, D. (1997) Crossreactions involving natural rubber latex. *Clin Rev Allergy Immunol* **15**, 429–447.
62. Brehler, R., Theissen, U., Mohr, C. & Luger, T. (1997) "Latex–fruit syndrome": frequency of cross-reacting IgE antibodies. *Allergy* **52**, 404–410.
63. Diaz-Perales, A., Collada, C., Blanco, C. *et al.* (1998) Class I chitinases with hevein-like domain, but not class II enzymes, are relevant chestnut and avocado allergens. *J Allergy Clin Immunol* **102**, 127–133.
64. Diaz-Perales, A., Collada, C., Blanco, C. *et al.* (1999) Cross-reactions in the latex–fruit syndrome: A relevant role of chitinases but not of complex asparagine-linked glycans. *J Allergy Clin Immunol* **104**, 681–687.
65. Yagami, T., Sato, M., Nakamura, A. *et al.* (1998) Plant defense-related enzymes as latex antigens. *J Allergy Clin Immunol* **101**, 379–385.
66. Yagami, T., Sato, M., Nakamura, A. & Shono, M. (1995) One of the rubber latex allergens is a lysozyme. *J Allergy Clin Immunol* **96**, 677–686.
67. Jaggi, K.J., Hovanec-Burns, D. & Unver, E. (1995) Existence of profilin in latex allergen. *J Allergy Clin Immunol* **95**, 212.
68. Vallier, P., Balland, S., Harf, R., Valenta, R. & Deviller, P. (1995) Identification of profilin as an IgE-binding component in latex from *Hevea brasiliensis*: clinical implications. *Clin Exp Allergy* **25**, 332–339.
69. Diez-Gomez, M.L., Quirce, S., Cuevas, M. *et al.* (1999) Fruit-pollen-latex cross-reactivity: implication of profilin (Bet v 2). *Allergy* **54**, 951–961.
70. Yip, L., Hickey, V., Wagner, B. *et al.* (2000) Skin prick test reactivity to recombinant latex allergens. *Int Arch Allergy Immunol* **121**, 292–299.
71. Nieto, A., Mazon, A., Boquete, M. *et al.* (2000) Assessment of profilin as an allergen for latex sensitized patients. *J Allergy Clin Immunol* **105**, S242.
72. Pastorello, E.A., Pravettoni, V., Ispano, M. *et al.* (1996) Identification of the allergenic components of kiwi fruit and evaluation of their cross-reactivity with timothy and birch pollens. *J Allergy Clin Immunol* **98**, 601–610.
73. Möller, M., Paschke, A., Vieluf, D., Kayma, M., Vieths, S. & Steinhart, H. (1997) Characterization of allergens in kiwi fruit and detection of cross-reactivities with allergens of birch pollen and related fruit allergens. *Food Agricult Immunol* **9**, 107–121.
74. Reindl, J., Rihs, H.P., Scheurer, S., Wangorsch, A., Haustein, D. & Vieths, S. (2002) IgE reactivity to profilin in pollen-sensitized subjects with adverse reactions to banana and pineapple. *Int Arch Allergy Immunol* **128**, 105–114.
75. Fuchs, T., Spitzauer, S., Vente, C. *et al.* (1997) Natural latex, grass pollen, and weed pollen share IgE epitopes. *J Allergy Clin Immunol* **100**, 356–364.
76. Quirce, S., Bombin, C., Aleman, A. & Sastre, J. (2000) Allergy to latex, fruit, and pollen. *Allergy* **55**, 896–898.
77. Kleber-Janke, T., Crameri, R., Appenzeller, U., Schlaak, M. & Becker, W.M. (1999) Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int Arch Allergy Immunol* **119**, 265–274.
78. Rihs, H.P., Chen, Z., Rueff, F. *et al.* (1999) IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes. *J Allergy Clin Immunol* **104**, 1293–1301.
79. Rihs, H.P., Rozynek, P., May-Taube, K., Welticke, B. & Baur, X. (1994) Polymerase chain reaction based cDNA cloning of wheat profilin: a potential plant allergen. *Int Arch Allergy Immunol* **105**, 190–194.
80. Fritsch, R., Ebner, H., Kraft, D. & Ebner, C. (1997) Food allergy to pumpkin seed – characterization of allergens. *Allergy* **52**, 335–337.
81. Jensen-Jarolim, E., Gerstmayer, G., Kraft, D., Scheiner, O., Ebner, H. & Ebner, C. (1999) Serological characterization of allergens in poppy seeds. *Clin Exp Allergy* **29**, 1075–1079.
82. Asturias, J.A., Arilla, M.C., Gomez-Bayon, N. *et al.* (1998) Cloning and immunological characterization of the allergen Hel a 2 (profilin) from sunflower pollen. *Mol Immunol* **35**, 469–478.

83. Fah, J., Wuthrich, B. & Vieths, S. (1995) Anaphylactic reaction to lychee fruit: evidence for sensitization to profilin. *Clin Exp Allergy* **25**, 1018–1023.
84. Reindl, J., Anliker, M.D., Karamloo, F., Vieths, S. & Wuthrich, B. (2000) Allergy caused by ingestion of zucchini (Cucurbita pepo): characterization of allergens and cross-reactivity to pollen and other foods. *J Allergy Clin Immunol* **106**, 379–385.
85. Anliker, M.D., Reindl, J., Vieths, S. & Wuthrich, B. (2001) Allergy caused by ingestion of persimmon (Diospyros kaki): detection of specific IgE and cross-reactivity to profilin and carbohydrate determinants. *J Allergy Clin Immunol* **107**, 718–723.
86. Vallverdu, A., Asturias, J.A., Arilla, M.C. *et al*. (1998) Characterization of recombinant Mercurialis annua major allergen Mer a 1 (profilin). *J Allergy Clin Immunol* **101**, 363–370.
87. Ledesma, A., Rodriguez, R. & Villalba, M. (1998) Olive-pollen profilin. Molecular and immunologic properties. *Allergy* **53**, 520–526.
88. Leitner, A., Vogel, M., Radauer, C. *et al*. (1998) A mimotope defined by phage display inhibits IgE binding to the plant panallergen profilin. *Eur J Immunol* **28**, 2921–2927.
89. Benitez, D., Garcia-Ortega, P., Picado, C. *et al*. (2001) Specific immune response to Phleum pratense plant profilin in atopic patients and control subjects. *Allergol Immunopathol* (Madr) **29**, 9–15.
90. Dormann, D., Montermann, E., Klimek, L. *et al*. (1997) Heterogeneity in the polyclonal T-cell response to birch pollen allergens. *Int Arch Allergy Immunol* **114**, 272–277.
91. Reekers, R., Busche, M., Wittmann, M., Kapp, A. & Werfel, T. (1999) Birch pollen-related foods trigger atopic dermatitis in patients with specific cutaneous T-cell responses to birch pollen antigens. *J Allergy Clin Immunol* **104**, 466–472.
92. Mari, A. (2001) Multiple pollen sensitization: a molecular approach to the diagnosis. *Int Arch Allergy Immunol* **125**, 57–65.
93. Venuti, A., Ricciuto, G.M., Romano, A., Marsigli, L., Stefanini, G.F. & Gasbarrini, G. (1997) Cross-reactivity between pollen and food allergens: the importance of Bet v 1 and Bet v 2 epitopes. *Int J Immunopathol Pharmacol* **10**, 71–75.
94. Pauli, G., Oster, J.P., Deviller, P. *et al*. (1996) Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: diagnostic value for birch pollen and associated allergies. *J Allergy Clin Immunol* **97**, 1100–1109.
95. Bircher, A.J., Van Melle, G., Haller, E., Curty, B. & Frei, P.C. (1994) IgE to food allergens are highly prevalent in patients allergic to pollens, with and without symptoms of food allergy. *Clin Exp Allergy* **24**, 367–374.
96. Daschner, A., Crespo, J.F. & Pascual, C.Y. (1998) Specific IgE to recombinant vegetal panallergen (rBet v 2) and fruit allergy in pollinic patients. *Allergy* **53**, 614–618.
97. Vidali, L., Perez, H.E., Valdes Lopez, V., Noguez, R., Zamudio, F. & Sanchez, F. (1995) Purification, characterization, and cDNA cloning of profilin from *Phaseolus vulgaris*. *Plant Physiol* **108**, 115–123.
98. Jankiewicz, A., Aulepp, H., Altmann, F., Fötisch, K. & Vieths, S. (1998) Serological investigation of 30 celery-allergic patients with particular consideration of the thermal stablity of IgE binding celery allergens. *Allergy J* **7**, 87–95.
99. Guex, N. & Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714–2723.
100. Willerroider, M., Fuchs, H., Ballmer-Weber, B. *et al*. Cloning and molecular and immunological characterization of two new food allergens, Lyc e 1 and Cap a 2, profilins from bell pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*). *Int Arch Allergy Immunol* (in press).

# 8 Bet v 1-Homologous Allergens

KARIN HOFFMANN-SOMMERGRUBER
and CHRISTIAN RADAUER

## 8.1 Introduction

Birch pollen (*Betula verrucosa*) is an important source of inhalant allergens in industrialised countries of the northern hemisphere, causing Type 1 allergic symptoms in atopic individuals. In the 1970s, several research teams identified around ten individual allergenic proteins from aqueous extracts of birch pollen, while in 1983, the 17 kDa major allergenic protein from birch pollen was purified by chromatographic and electrophoretic methods, and was given the allergen name Bet v 1 [1] .

The complete coding sequence for Bet v 1, representing one of the first cloned allergens, was determined in 1989. Originally, it was identified by screening a pollen cDNA library with serum IgE from allergic patients [2]. About 90% of birch pollen-allergic patients display specific IgE antibodies directed against Bet v 1, of which 70% are monosensitised to Bet v 1 [3].

Comparison of the nucleotide sequence of the cloned allergen to the sequences from the EMBL databank known at that time revealed a sequence similarity of 70% to a pea disease-resistance response gene (*Pisum sativum*) [4]. These proteins are designated pathogenesis-related (PR) proteins, proteins that are expressed upon pathogen attack, stress and abiotic stimuli. However, a number of isoforms related to PR-proteins are also expressed constitutively in certain developmental stages and/or tissues. The latest classification of PR-proteins by Van Loon, from 1999, has allocated Bet v 1 homologues to the family PR10 (PR family of unknown function, also termed as intracellular pathogenesis-related proteins, IPR [5]). The widespread occurrence and the conservation of these proteins within the plant kingdom indicate an important role. Most of the genes for PR proteins have been shown to be induced upon microbial attack [6], fungal elicitors [6, 7], wounding [8] or stress stimuli [9].

Based on clinical observations, it has been known for a long time that birch pollen-allergic patients tend to display allergic symptoms when eating apples, pears, nuts, carrots, potatoes, celery and many other plant-derived foods [10–13]. The first data on cross-reactivity based on homologous structures present in birch pollen as well as in apples (*Malus domestica*) were published by Ebner and co-workers [14]. The authors used western and northern blotting techniques to demonstrate that apples harbour proteins with high homology to Bet v 1. The presence of Bet v 1-related structures was further investigated in immunoblots performed with apple, pear, celery, carrot and potato extracts. Allergic patients' sera and monoclonal Bet v 1 antibodies recognised homologous proteins present in these fruit and vegetable extracts [15].

## 8.2 Physico-chemical properties of Bet v 1 homologues

Originally, Bet v 1 homologues were described as allergenic proteins from pollen of birch and related trees such as hazel (*Corylus avellana* [16]), alder (*Alnus glutinosa* [17]), hornbeam (*Carpinus betulus* [18]) and European chestnut (*Castanea sativa* [19]). Since then, a growing number of small intracellular proteins with similar characteristics have been described from a wide range of flowering plants, including dicots as well as monocots. The presence of these highly conserved sequences in a great number of even distantly related species may point to a crucial function of this protein family.

### 8.2.1 *Sequences and genomic organisation*

Bet v 1 homologues contain open reading frames from 465 bp to 480 bp. Bet v 1 genes consist of either one or two exons interrupted by an intron [20]. Comparison of a number of genomic sequences of the Bet v 1 family shows that the intron position is highly conserved among sequences derived from different dicot species (amino acid position 62 for parsley (*Petroselinum crispum*, PcPR1), hazel (C*orylus avellana*, cagc11) and birch (*B. verrucosa*, bvgc70), codon 61 for pea (*Pisum sativum*, PI-49) and codon 57 for potato (*Solanum tuberosum*, STH-2). Even the monocot asparagus' (*Asparagus officinale*) AoPR1 gene contains an intron at position 63 [20].

Various isoforms of Bet v 1 homologues are expressed within one species. Southern blots performed with *B. verrucosa* DNA suggested the presence of numerous Bet v 1 genes (I. Swoboda, personal communication). In addition, purified Bet v 1 separated into several spots on two-dimensional gels [21, 22]. Subsequently, a number of isoforms of Bet v 1 were identified by RT–PCR, and the expression of several of these isoforms was verified at the amino acid level by mass spectrometry [23]. These isoforms are highly homologous with amino acid sequence identities ranging from 84 to 99% [23]. A different subset of Bet v 1 sequences, designated Bet v 1-Sc1–Bet v 1-Sc3, was identified from birch callus upon co-cultivation with microbial pathogens [24, 25]. These three clones are highly homologous to, but different from the pollen isoforms. The genes coding for Bet v 1-Sc1–3 have been isolated from a birch genomic library and designated *Ypr10*a*, *Ypr10*b* and *Ypr10*c* [26]. These genes have been found to be clustered in the genome, within 14 kb of DNA.

Bet v 1-Sc1 and Bet v 1-Sc2 are nearly identical, and could be the allelic variants of the same gene; whereas, Bet v 1-Sc3 is different from Sc1 and Sc2. Bet v 1-Sc1 and Bet v 1-Sc3 display 73% sequence identity at the amino acid level. The sequence identities of Bet v 1-Sc proteins to the various pollen isoforms range 70–74% in the case of Bet v 1-Sc3, and 83–88% in the case of Bet v 1-Sc1 and Sc2.

A number of isoforms with high sequence identity to each other have been isolated from Mal d 1, the Bet v 1 homologue from apple [27]. Furthermore, 20 different sequences derived from different apple cultivars, with a length of 159 amino acids, are presently in the sequence databases. Api g 1.0101, encoding a protein of 154 amino acid residues, was the first Bet v 1 homologue identified from celery (*Apium graveolens* [28]). A second isoform, Api g 1.0201, which is five amino acid residues longer at the *C*-terminus, and shares only 52% sequence identity with Api g

1.0101 has been isolated from celery by screening a cDNA library with allergic patients' sera [29]. Api g 1.0201 is more closely related to PCPR1–2, the PR 10 from parsley, than to Api g1.0101.

Phylogenetic analyses performed with 67 Bet v 1-homologous sequences derived from 22 species and 7 plant families are generally consistent, with those based on plant morphology and other biochemical markers [30].

So far, no members of the PR10 family have been identified from tobacco (*Nicotiana tabacum*). Recently, major latex proteins (MLPs), a family with unknown function, were found in the latex from opium poppy (*Papaver somniferum*), arabidopsis (*Arabidopsis thaliana*), bell pepper (*Capsicum annuum*), melon (*Cucumis melo*), tobacco (*Nicotiana tabacum*) and strawberry (*Fragaria vesca*) [31]. In some cases these proteins have been found to be induced upon wounding. Comparison of the three-dimensional structure of a Bet v 1 isoform, with the predicted secondary structure of MLPs, showed striking structural similarity, although the sequence identity is less than 25%. These data could point to a relationship of these two protein families, as well as to similar functions of both groups of proteins within the plant.

### 8.2.2 *Expression patterns of Bet v 1 homologues*

Pathogenesis-related proteins of family 10 are induced upon microbial attack, fungal elicitors, wounding, or other physical or chemical stress. However, they are also constitutively expressed in some organs or during certain developmental stages. Such PR10 proteins have been isolated from various species such asparagus [8], parsley [6], bean [7], potato [32] and apple [33].

8.2.2.1 *Constitutive expression of Bet v 1 homologues.* Bet v 1 expression is usually restricted to certain developmental stages and tissues of birch. Bet v 1 proteins are present in high quantities in mature pollen and male inflorescences, and accumulate in old leaves. In contrast to these observations, *in vitro* translation of RNA from all tissues as well as from birch callus yielded detectable levels of Bet v 1, suggesting that Bet v 1 can be readily synthesised in tissues other than pollen or old leaves [34], and that regulation of protein synthesis of Bet v 1 occurs at translational level. A similar situation was found when the Bet v 1 homologue from apple, Mal d 1, was characterised [33]. This protein is constitutively expressed in ripe apples as well as in old leaves, but is not detectable in apple pollen.

Abscisic acid (ABA) and ethylene are plant hormones important for developmental regulation. In pea, the synthesis of PR10 proteins is induced by ABA in late seed development [35], whereas a PR10 protein from asparagus is predominately expressed in developing seeds [36]. The authors suggest that the PR10 protein synthesis correlates with the synthesis of enzymes of the core phenylpropanoid pathway, which leads to the synthesis of cell-wall structural molecules, pigments, UV-light protectants, chemical attractants and antimicrobial phytoalexins. Along the same lines, it was proposed by Yazaki and co-workers that the LEDI-1 protein from *Lithospermum erythrorhizon* might play a role in the induction of the secondary metabolism such as shikonin production [37].

In yellow lupin (*Lupinus luteus*), two PR10 protein isoforms (L1PR101A and L1PR101B; 91% similarity) were identified [38]. Both proteins are constitutively expressed in roots and stem. In addition, L1PR101B is constitutively expressed in leaf and petiole, while L1PR101A is induced in senescent leaves.

In the gymnosperm pine (*Pinus pinaster*) a PR10 protein of 150 amino acid residues was detected in needles after drought stress [39]. Roots of dandelion (*Taraxacum officinale*) contain a PR10 protein, consisting of two major isoforms, that undergoes seasonal fluctuations in amount, increasing in the late autumn months and declining in spring [40]. This predominant 18 kDa-root protein has been suggested to represent either a vegetative storage protein or a low temperature responsive gene product, involved in the cold protection of the plant, but evidence for these hypotheses is lacking so far.

8.2.2.2 *Induced expression of Bet v 1 homologues.* A subset of Bet v 1 proteins, Bet v 1-Sc1–3, was identified in birch callus cultures upon co-cultivation with microbial pathogens [24, 25]. Utriainen and co-workers demonstrated that copper and ozone induced the expression of Bet v 1-Sc1–3 in roots and leaves of birch seedlings [41].

Mal d 1 mRNA and protein levels were increased in young apple leaves upon induction with fungal elicitors [33]. Also, treatment with salicylic acid (SA), known to be a mediator of signal transduction in pathogen defense and stress response, resulted in upregulation of the Mal d 1 sequences, as did the addition of reduced glutathione (GSH), a substance known to be involved in oxidative stress and selective induction of PR-proteins [42]. In contrast, ABA did not show any effect on the Mal d 1 expression level [33].

PR10 proteins were identified in roots of Leguminosae, after mite attack [43] and in the early stage of interactions with microsymbiontic bacteria [44]. Similarly, rapid accumulation of a PR10 protein, as a resistance response to a non-pathogenic fungus, was observed in sorghum (*Sorghum bicolor* [45]).

8.2.2.3 *Promoter structures of Bet v 1 homologues.* Taken together, it seems that the Bet v 1 gene family consists of various, differently regulated members including genes that are expressed constitutively in pollen and old leaves, and another subset of genes that is induced upon pathogen attack and by stress factors.

A Mal d 1 promoter (Ypr10*) was isolated, and induction experiments performed in tobacco plants [33]. The data on promoter activity were in good agreement with the results gained from the expression studies of Mal d 1 in leaves. Reporter gene constructs showed induced activity upon treatment with either GSH or SA. However, a putative SA-inducible consensus motif was absent from the promoter sequence. ABA and etephon did not influence Ypr10*a activity. Pathogens, like tobacco mosaic virus, tobacco etch virus, tobacco vein mottling virus and secreted cell wall elicitors from *Botrytis cinerea* also induced Mal d 1 promoter activity in transgenic plants.

More detailed investigations are needed to find out whether several differentially regulated Ypr10* promoters are present in the apple genome. This will clarify whether Mal d 1 expression is regulated via different promoters or via a common promoter induced in response to different signals.

The first hypothesis would suggest the presence of different promoters, leading to constitutive expression under developmental regulation versus promoters induced upon stress factors.

The second hypothesis would postulate the existence of several converging signal transduction pathways leading to the activation of a type of Ypr10* promoter.

8.2.2.4 *Biochemical function of Bet v 1 homologues.* Up to now, no distinct biochemical function of PR10 proteins has been shown. There is a striking conserved sequence motif GXGXXG at amino acid residues 47–52 (Fig. 8.1) throughout the Bet v 1 family (except in STH-2 where it is reduced to GXG). This motif is flanked by conserved lysine residues – 14 amino acid residues upstream and 17 amino acid residues downstream (except for Api g 1 and Dau c 1, which lack the downstream

```
           1                                    *                50
Bet v 1a   MGVFNYETET TSVIPAARLF KAFILDGDNL FPKVAPQAIS SVENIEGNGG
Api g 1    MGVQTHVLEL TSSVSAEKIF QGFVIDVDTV LPKAAPGAYK SVE.IKGDGG
Dau c 1.2  MGAQSHSLEI TSSVSAEKIF SGIVLDVDTV IPKAATGAYK SVE.VKGDGG
Pyr c 1    MGLYTFENEF TSEIPPPRLF KAFVLDADNL IPKIAPQAIK HAEILEGNGG
Mal d 1    MGVYTFENEF TSEIPPSRLF KAFVLDADNL IPKIAPQAIK QAEILEGNGG
Pru av 1   MGVFTYESEF TSEIPPPRLF KAFVLDADNL VPKIAPQAIK HSEILEGDGG
Consensus  **------*- **-------* -----*-*-- -**-*--*-- --*---*-**

           51                  *                               100
Bet v 1a   PGTIKKISFP EGFPFKYVKD RVDEVDHTNF KYNYSVIEGG PIGDTLEKIS
Api g 1    PGTLKIITLP DGGPITTMTL RIDGVNKEAL TFDYSVIDGD ILLGFIESIE
Dau c 1.2  AGTVRIITLP EGSPITTMTV RTDAVNKEAL SYDSTVIDGD ILLGFIESIE
Pyr c 1    PGTIKKITFG EGSQYGYVKH RVDSIDEASY SYAYTLIEGD ALTDTIEKIS
Mal d 1    PGTIKKITFG EGSQYGYVKH RIDSIDEASY SYSYTLIEGD ALTDTIEKIS
Pru av 1   PGTIKKITFG EGSQYGYVKH KIDSIDKENY SYSYTLIEGD ALGDTLEKIS
Consensus  -**---*--- -*-------- --*------- ------*-*- ------*-*-

           101                                             150
Bet v 1a   NEIKIVATPD GGSILKISNK YHTKGDHEVK AEQVKASKEM GETLLRAVES
Api g 1    NHVVLVPTAD GGSICKTTAI FHTKGDAVVP EENIKYANEQ NTALFKALEA
Dau c 1.2  THMVVVPTAD GGSITKTTAI FHTKGDAVVP EENIKFADAQ NTALFKAIEA
Pyr c 1    YEAKLVASGS .GSTIKSISH YHTKGDIEIK EEHVKAGKEK AHGLFKLIES
Mal d 1    YETKLVACGS .GSTIKSISH YHTKGNIEIK EEHVKAGKEK AHGLFKLIES
Pru av 1   YETKLVASPS GGSIIKSTSH YHTKGNVEIK EEHVKAGKEK ASNLFKLIET
Consensus  -----*---- -**--*---- -****----- -*--*----- ---*----*-

           151    160
Bet v 1a   YLLAHSDAYN
Api g 1    YLIAN.....
Dau c 1.2  YLIAN.....
Pyr c 1    YLKDHPDAYN
Mal d 1    YLKDHPDAYN
Pru av 1   YLKGHPDAYN
Consensus  **--------
```

**Fig. 8.1** Alignment of amino acid sequences of Bet v 1 homologues compared to Bet v 1a (conserved amino acid residues are marked by an asterisk). A conserved motif (P-loop) is marked (shaded region) and the flanking lysine residues are marked. Bet v 1a (EMBL database, accession no: X15899); Api g 1 (accession no: Z48967); Dau c 1.2 (accession no: Z81361); Pyr c 1 (accession no: AF 057030); Mal d 1 (accession no: AJ417551); Pru av 1 (accession no: U66076).

lysine residue). This motif is known as a P-loop (phosphate-binding loop), and is frequently found in protein kinases as well as in nucleotide-binding proteins [46].

As yet, there are no experimental data corroborating a nucleotide-binding function. Firstly, there is no overall pattern common for all ATP- or GTP-binding protein P-loops. Secondly, homology based exclusively on this sequence motif does not necessarily mean that the candidate protein must have a kinase activity. The crystal structure of a Bet v 1 isoform [47] showed that the molecule contains a large forked cavity, that runs through its structure, with three openings on the surface. The P-loop is in close vicinity to this cavity. Neudecker and co-workers resolved the structure of the Bet v 1-homologue from cherry, Pru av 1 (*Prunus avium*, [48] Plate 8.1). Both the secondary structure elements and the tertiary fold of these two allergens are virtually identical. Surprisingly, there is a striking structural homology to the START domain of the human protein MLN64 over 129 amino acid residues, although sequence identity is only 8.5%. START domains are known to be associated with the transfer of lipids, especially steroids. In parallel, experiments with Pru av 1 demonstrated that homocatasterone (brassinolid) binds to Pru av 1. These results are in good agreement with the experiments performed with Bet v 1, where the binding of plant steroids in the cavity of the Bet v 1l structure was also demonstrated (M. Degano, personal communication).

Nevertheless, it is still not known whether one or two steroid molecules bind in this cavity, and which particular steroids. Furthermore, it is also not known whether this binding occurs *in vivo*, and if so, what is the physiological role?

Ribonuclease activity has been shown for PR10 proteins from ginseng (*Panax panax* [49]), and this activity has also been demonstrated for Bet v 1 [50, 51] in *in vitro* assays.

To date, the designation of the PR10 family – proteins with unknown function – holds true. Since the PR10 protein family is highly conserved throughout dicotyledonous plants and also occurs in monocots, gymnosperms [39] and mosses [52], a crucial function can be assumed in the plant.

## 8.3 Allergological features of Bet v 1 homologues

### 8.3.1 *Bet v 1-related food allergies of the oral allergy syndrome type*

In northern and central Europe, tree pollen allergy is mainly caused by pollen allergens from birch and related trees of the order Fagales. As a consequence of an allergic sensitisation to inhalant allergens, tree pollen-allergic patients can develop food allergies. Cross-reactive IgE is the immunological basis for these food allergies [15], but the presence of food allergen-specific IgE is not always accompanied by clinical symptoms [53].

More than 50% of birch pollen-allergic patients show allergic symptoms towards various plant-derived foods. In the majority of cases, the allergen responsible for this cross-reactivity is Bet v 1. This type of food allergy is usually confined to the oral allergy syndrome (OAS), with symptoms usually comprising local reactions of the mucosa of the upper aero-digestive tract with itching, inflammation and angioedema.

### 8.3.2 *Sequence data*

Based on clinical observations, it has been known for some time that birch pollen-allergic patients tend to display allergic symptoms when eating apples, pears, nuts carrots, potatoes, celery and many other plant-derived foods [10–13]. The first data on cross-reactivity based on homologous structures present in birch pollen as well as in apples were published by Ebner and co-workers [14]. The authors used western and northern blotting techniques to demonstrate that apples harbour proteins with high homology to Bet v 1. The presence of Bet v 1-related structures was further investigated in immunoblots performed with apple, pear, celery, carrot and potato extracts. Allergic patients' sera and monoclonal anti-Bet v 1 antibodies recognised the homologous proteins present in these fruit and vegetable extracts [15].

In the past few years, a number of cDNA sequences coding for Bet v 1-homologous food allergens have been isolated, cloned and sequenced. Mal d 1, the Bet v 1-related apple allergen, was the first food allergen to be cloned, characterised and produced as a recombinant protein [54]. Other cloned fruit allergen sequences include cDNAs encoding Pru av 1 from sweet cherry [55], Pru ar 1 from apricot (*Prunus armeniaca*, Pühringer EMBL access no: AF020784; Mbeguie-Mbegiue, access no: AF134731) and Pyr c 1 from pear (*Pyrus communis* [56]). Recombinant proteins have also been produced from the Bet v 1 homologues from celery (Api g 1 [28]), and carrot (*Daucus carota* Dau c 1 [57]). In addition, Bet v 1-homologous proteins with binding capacity to anti-Bet v 1-IgE have been described as pcPR1 and pcPR2 from parsley, and as pSTH2 and pSTH21 from potato [20].

Bet v 1-related sequences from apple, cherry, celery and carrot encode proteins of 154–160 amino acid residues (including the initiating methionine), and show sequence identities ranging 44–88% (Fig. 8.2).

Sequence identities among the fruit (Rosaceae 87–88%) and vegetable (Apiaceae 82%) allergens are significantly higher than their identities to Bet v 1 (Rosaceae: Bet v 1 64–66%, Apiaceae: Bet v 1 44% Fig. 8.2), reflecting their phylogenetic relationships.

### 8.3.3 *Clinical features of Bet v 1 homologues*

Bet v 1-related proteins are rather heat-sensitive and protease-labile. Incubation of Mal d 1 and Bet v 1 with digestive enzymes mimicking the gastrointestinal tract led within seconds to a degraded protein, that did not recognise cross-reactive monoclonal anti-Bet v 1 antibodies [58, 59]. Their sensitivity against proteases explains why Bet v 1 homologues primarily evoke allergenic reactions in the oral mucosa and do not frequently cause generalised or gastro-intestinal problems. In addition, plant food that is eaten after thermal processing does not represent a significant source of allergen for Bet v 1-allergic patients.

In the birch–apple syndrome, the OAS is predominant, whereas in the birch–celery–mugwort–spice syndrome, anaphylactic shock has also been reported. In the latter syndrome, at least three distinct allergenic structures have been identified so far: profilin, Api g 1 and higher molecular weight allergens in the range of 40–69 kDa [11, 60]. These higher molecular weight allergens represent very stable food allergens.

| Bet v 1 | Api g 1 | Dau c 1.2 | Pyr c 1 | Mal d 1 | Pru av 1 | |
|---|---|---|---|---|---|---|
| 100 | 44 | 44 | 64 | 64 | 66 | Bet v 1 |
| | 100 | 82 | 46 | 48 | 50 | Api g 1 |
| | | 100 | 45 | 45 | 47 | Dau c 1.2 |
| | | | 100 | 88 | 87 | Pyr c 1 |
| | | | | 100 | 88 | Mal d 1 |
| | | | | | 100 | Pru av 1 |

**Fig. 8.2** Sequence comparison among the Bet v 1-homologous allergens. Sequence identities are given on the amino acid level; Bet v 1a (EMBL database, accession no: X15899); Api g 1 (accession no: Z48967); Dau c 1.2 (accession no: Z81361); Pyr c 1 (accession no: AF 057030); Mal d 1 (accession no: AJ417551); Pru av 1 (accession no: U66076) [88].

Even after heating to 100 °C for 30 minutes [61], the IgE binding was still detectable in a range of 30–69 kDa, whereas the IgE binding to Api g 1 was completely destroyed.

Allergic cross-reactivity based on Bet v 1-homologous proteins seems to be restricted to areas where birch trees are common. For example, sensitisation to Api g 1 happens frequently in celery-allergic patients living in northern and central Europe, where birch trees are common. In areas where birch is rare, e.g. southern parts of Europe, sensitisation to Api g 1 could not be observed in celery–mugwort-allergic individuals [62]. This is consistent with the hypothesis that inhalant pollen is the primary sensitising agent in Bet v 1-related allergies.

However, exceptions have also been reported; Moneo and co-workers described four Spanish carrot-allergic patients with specific IgE directed to Dau c 1, which cross-reacts with Api g 1 but without cross-reactivity to Bet v 1 [63].

### 8.3.4 *Individual Bet v 1-related food allergens derived from different botanical families, their cross reactivities and allergological importance*

#### 8.3.4.1 *Rosaceae*

8.3.4.1.1 *Apple (Malus domestica).* Mal d 1 was the first cloned Bet v 1-related food allergen [64], followed shortly by other Mal d 1 isoforms [65, 66]. For example, two studies revealed adverse reaction to apples in 24/51 [67] and 63/83 [14] of birch pollen-sensitised patients, respectively. Comparative testing of purified recombinant Bet v 1 and Mal d 1 revealed that IgE binding to Mal d 1 was abolished by preincubation of allergic patients' sera with Bet v 1, whereas preincubation with Mal d 1 only reduced IgE binding to Bet v 1.

Differences in the allergenic potency among different apple strains have been reported by Vieths and co-workers [68]. Recently, a number of Mal d 1 isoforms have been isolated, cloned and sequenced, and 20 different sequences encoding Mal d 1 are present in the sequence databases. Son and co-workers investigated 13 different isoforms derived from seven different cultivars [27]. Based on sequence similarities, they identified six clones belonging to the Mal d 1a family (identities of 97.5–94%), six clones with similarity to the Mal d 1b family (99.4–100%) and another isoform, Mal d 1c, with minor frequency. The authors concluded that the occurrence of Mal d 1 isoallergens is not cultivar-specific, and that a mixture of isoforms is present in the apple fruit. As a consequence, the allergenic potency of different cultivars is related to the total level of expressed Mal d 1 isoforms.

Comparison of Bet v 1 isoforms shows that a restricted number of amino acid exchanges at special hot spots can change the IgE-binding activity of the proteins from very strong to very weak [69]. Based on these findings, Ferreira and co-workers performed *in vitro* mutagenesis with Mal d 1 and showed that amino acid positions 10, 30, 57, 112 and 113 are critical for the IgE binding [70]. Son and co-authors similarly demonstrated that position 111 is a hot spot for IgE binding [27]. Based on the resolved structure of the homologous cherry and birch allergens, Pru av 1 and Bet v 1, this key residue is the first in a β-sheet and is exposed on the surface [47, 48].

The influence of storage and ripening conditions of apples on the expressed level of Mal d 1 has not yet been investigated in detail. Preliminary data from Hsieh and co-workers [71] suggest that the Mal d 1 content increases during prolonged storage at 4°C. However, the level of the Bet v 1 homologue in apples is not elevated during the ripening processes induced by ethylene treatment. Hopefully, further detailed investigations on storage conditions and post-harvest treatments may help to develop strategies to reduce the allergen load from apples.

The cross-reactivity between Bet v 1 and Mal d 1 can be demonstrated not only by IgE antibodies with binding capacity to both allergens, but also at the level of allergen-specific T helper cells [72]. Allergen-specific T-cell lines and T-cell clones were established from peripheral blood from birch pollen–apple-allergic patients. Bet v 1- and Mal d 1-specific T-cell cultures were further epitope mapped using overlapping peptides. The majority of cross-reactive T-cell clones revealed a $TH_2$-like cytokine production pattern, and six cross-reactive T-cell epitopes with approximately 50% amino acid identity were identified. It seems that Bet v 1 homologues possess the capacity to activate Bet v 1-specific T-lymphocytes and therefore possibly provide help to maintain IgE production outside the pollen season [73]. Bet v 1-homologous food allergens may also play a direct role in the sensitisation process by priming T cells which then react with pollen proteins.

8.3.4.1.2 *Mal d 1-homologous allergens from the Rosaceae family.* Mal d 1-related sequences have been identified from pear (Pyr c 1 [56]), cherry (Pru av 1 [55]) and apricot (Pühringer, EMBL: accesss no.AF020784, MbeguieA-Mbeguie D. AF134731). Recombinant proteins have been produced from pear and cherry, and their allergenic activity was tested in immunoblots, inhibition assays and basophil histamine release assays [55, 56].

In southern Europe, allergies to fruits of the family Rosaceae are not caused by the recognition of Bet v 1-homologous proteins from the immune system. Since birch trees are practically not growing in this climate, the primary sensitising pollen agent is not present. In these countries, food allergies against stone fruits are caused by different allergens like the lipid transfer protein (LTP), and are often associated with rather severe food-allergic symptoms [74, 75].

#### 8.3.4.2 *Apiaceae*

8.3.4.2.1 *Celery (*Apium graveolens*).* Api g 1.0101, a protein of 154 amino acid residues, was the first Bet v 1 homologue identified from celery [28]. In cross-inhibition assays, recombinant Bet v 1a is able to block any IgE binding to natural Api g 1, whereas preincubation of the allergic patients' sera with recombinant Api g 1 does not inhibit IgE binding to natural Bet v 1. These data are in agreement with other cross-inhibition assays between Bet v 1-related food allergens and Bet v 1a, showing that Bet v 1-related proteins share common epitopes and, in addition, Bet v 1a possesses additional epitopes which cannot be inhibited with Mal d 1, Api g 1 or Dau c 1. The six hot spots known from Bet v 1a to be critical for IgE binding are also valid for Api g 1.0101. After exchanging amino acids in positions 10, 30, 57, 112, 113 and 125, the IgE-binding activity of the mutant was significantly reduced, compared to wild-type Api g 1.0101 [70].

The sequence encoding Api g 1.0101 encompasses an open reading frame of 154 amino acid residues. The sequence resembles those of other PR10 proteins from the Apiaceae family, and the P-loop is different from those of other Bet v 1 homologues by a negatively charged Glu45 being substituted by a positively charged lysine. Recently, another isoform has been isolated from celery by screening a cDNA library with allergic patients' sera. Api g 1.0201 is five amino acid residues longer at the *C*-terminus and shares only 52% sequence identity with Api g 1.0101 [29]. Its sequence is clearly more related to that of PCPR1–2, a PR 10 protein from parsley. Api g 1.0201 also has the lysine substitution at position 45 in the P-loop, that is present in Api g 1.0101. These sequence differences are also reflected in the antibody-recognition pattern of Api g 1.0201; Api g 1.0201 does bind to IgE antibodies from celery-allergic patients' sera, but with less binding intensity compared to Api g 1.0101. Furthermore, preincubation with rBet v 1a does not inhibit IgE binding to Api g 1.0201, but natural Bet v 1, consisting of a mixture of isoforms, blocks IgE binding to Api g 1.0201. Finally, the monoclonal anti-Bet v 1–antibody BIP1, which has very weak cross-reactivity to Mal d 1 and Api g 1.0101, binds to Api g 1.0201 [29]. However, the sequence differences responsible for these different binding patterns remain to be elucidated.

Purified rApi g 1.0101 was tested in immunoblots and skin prick tests, using celery-allergic patients from central Europe and southern France [62]. Api g 1.0101 proved to be a valuable tool for diagnosis of birch–celery allergy in the group of patients from central Europe. However, Api g 1.0101 was not recognised by either *in vitro* or *in vivo* testing in celery-allergic patients living in the southern parts of Europe. In these areas, celery-allergic patients do not have birch pollen allergy, and are sensitised against different types of allergens.

8.3.4.2.2 *Carrot (*Daucus carota*).* Three closely related isoforms of 154 amino acid residues, Dau c 1.1–Dau c 1.3, have been isolated and characterised as Bet v 1 homologues from carrot [57] with 96–99% sequence identity with each other, and 81% identity to Api g 1. Two of these isoforms, Dau c 1.2 and Dau c 1.3 have been investigated in immunoblots, with a number of allergic patients' sera, and displayed no significant differences in their IgE-binding capacity. In cross-inhibition assays, preincubation with Api g 1.0101 significantly weakened IgE binding to nDau c 1, as well as rBet v 1a.

In central Europe, up to 25% of food-allergic subjects have carrot allergy [76]. In a recent study, Ballmer-Weber and co-workers confirmed carrot allergy in 20/26 patients by double-blind placebo-controlled food challenge (DBPCFC [77]). In 85% of the carrot-allergic patients, Dau c 1-specific IgE antibodies were detected in immunoblots. All these patients were also sensitised to rBet v 1. However, in a subset of patients ($n=4$), IgE binding to Dau c 1 was inhibited neither by rBet v 1 nor by birch pollen extract. These data would point to a sensitisation to carrot allergens, independent of a primary sensitisation to birch pollen allergens. Similar data have been reported from Moneo and co-workers. In four carrot-allergic patients, a monosensitisation to an 18 kDa protein in carrot was detected, without concomitant birch pollen sensitisation [63].

8.3.4.2.3 *Allergens from spices of the Apiaceae family.* The Bet v 1 homologue from parsley is able to bind IgE from a serum pool of birch pollen-allergic patients. However, the allergen has neither been purified nor cloned [20].

In a group of 15 spice-allergic patients with tree pollen allergy and/or mugwort allergy, the IgE binding to proteins from anise, fennel, cumin (*Carum carvi*) and coriander (*Coriandrum sativum*) was investigated [78]. IgE binding to a 17 kDa protein present in anise and fennel was observed in sera from six patients. Two of them also recognised homologous proteins present in coriander and cumin. Inhibition assays performed with rBet v 1a abolished IgE binding to these spice-derived proteins, and anti-Bet v 1 antibodies also cross-reacted with them, suggesting that Bet v 1 homologues are present in these spices and contribute to food-allergic reactions in celery–mugwort–birch-allergic patients.

## 8.4 Discussion and outlook: new concepts for specific immunotherapy using Bet v 1 as a model

Bet v 1 represents a good model for studying and evaluating improved diagnostic tools, and developing new immunotherapeutic strategies. In the following, only a few interesting examples will be presented.

So far, *in vitro* diagnosis of type 1 allergy has been performed with crude allergen extracts prepared from the allergen sources. Such crude allergen extracts contain, in addition to the desired allergens, a large variety of proteins, carbohydrates and nucleic acids, which are not allergenic. Certain allergens are either not well represented or degraded during the preparation procedure. Therefore, there is a great need for well-defined, standardised extracts, or purified individual allergens. Purified-recombinant

allergens, with allergenic activity equivalent to that of their natural counterparts, would improve diagnosis and could contribute to a patient-tailored immunotherapy. Bet v 1 has proved, in the past five years, to be a good diagnostic tool not only for *in vitro* diagnosis but also for *in vivo* testing [67, 79]. This has also been shown for Api g 1 which was used for skin prick tests in birch pollen–celery-allergic patients [62].

The spectrum of the relevant allergens, of an allergen source, has to be carefully investigated for diagnosis. Furthermore, the isoform with the highest IgE-binding capacity should be selected.

Specific immunotherapy of type 1 allergy is based on the systemic application of increasing doses of the allergens to which the patient is sensitised [80].

Currently, immunotherapy is performed with crude allergen extracts which contain mixtures of allergens and additional non-allergenic material. Purified, well-standardised recombinant allergens would represent a better choice for patient-tailored immunotherapy, where only one or a few allergens are administered [81]. Ferreira and co-workers approached this by using low IgE-binding isoforms of Bet v 1, which represent the full repertoire of the possible T-cell epitopes, but reduce the risk of anaphylactic side effects during immunotherapy [69, 82]. Vrtala and co-workers selected Bet v 1 fragments (fragment 1: aa 1–74, fragment 2: aa 75–160) as candidates for new immunotherapeutic strategies [83]. These two fragments exhibit a random coiled conformation, and do not bear the conformational IgE epitopes of Bet v 1, but mostly, dominant T-cell epitopes are present. These fragments showed significantly reduced ability to evoke a reaction in allergic patients in skin prick tests, when compared to the complete molecule of Bet v 1 [83, 84]. The authors could also show that by destroying the IgE epitopes of this molecule, they could induce the production of protective IgG antibodies in mice and rats upon immunisation with these hypoallergenic fragments of Bet v 1 [85].

Protective IgG antibodies were also induced in BALB/c mice, when mimotopes of Bet v 1 were injected [86, 87]. These peptides were isolated from a phage display peptide library, representing artificial conformational IgE epitopes. It can be hypothesised that these antibodies interfere with the IgE–allergen interaction, by inducing an IgG immune response in mice, and thus prevent further allergic reactions.

However, detailed investigations are needed in the future for these interesting new immunotherapies with Bet v 1. In addition, further clinical trials will verify whether these therapeutical concepts also contribute to amelioration of the related food-allergic symptoms.

## References

1. Ipsen, H. & Lowenstein, H. (1983) Isolation and immunochemical characterization of the major allergen of birch pollen (Betula verrucosa). *J Allergy Clin Immunol* **72**, 150–159.
2. Breiteneder, H., Pettenburger, K., Bito, A. *et al*. (1989) The gene coding for the major birch pollen allergen Bet v1, is highly homologous to a pea disease resistance response gene. *Embo J* **8**, 1935–1938.
3. Jarolim, E., Rumpold, H., Endler, A.T. *et al*. (1989) IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of Betula verrucosa. *Allergy* **44**, 385–395.
4. Fristensky, B., Horovitz, D. & Hadwiger, L. (1988) cDNA sequences for pea disease resistance response genes. *Plant Mol Biol* **11**, 713–715.

5. van Loon, L. & van Strien, E. (1999) The families of pathogenesis-related proteins, their acitivities, and comparative analysis of PR-1 type proteins. *Phys Mol Plant Pathol* **55**, 85–87.
6. Somssich, I., Schmelzer, E., Bollmann, J. & Hahlbrock, K. (1986) Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells. *Proc Natl Acad Sci USA* **83**, 2427–2430.
7. Walter, M., Liu, J., Grand, C., Lamb, C. & Hess, D. (1990) Bean pathognesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen alleregens. *Mol Gen Genet* **222**, 353–360.
8. Warner, S., Scott, R. & Draper, J. (1992) Characterisation of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. *Plant Mol Biol* **19**, 555–561.
9. Walter, M.H., Liu, J.W., Wunn, J. & Hess, D. (1996) Bean ribonuclease-like pathogenesis-related protein genes (Ypr10) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression. *Eur J Biochem* **239**, 281–293.
10. Eriksson, N.E., Formgren, H. & Svenonius, E. (1982) Food hypersensitivity in patients with pollen allergy. *Allergy* **37**, 437–443.
11. Wuthrich, B., Stager, J. & Johansson, S.G. (1990) Celery allergy associated with birch and mugwort pollinosis. *Allergy* **45**, 566–571.
12. Halmepuro, L., Vuontela, K., Kalimo, K. & Bjorksten, F. (1984) Cross-reactivity of IgE antibodies with allergens in birch pollen, fruits and vegetables. *Int Arch Allergy Appl Immunol* **74**, 235–240.
13. Van Ree, R. & Aalberse, R. (1993) Pollen-vegetable food crossreactivity: serological and clinical relevance of crossreactive IgE. *J Clin Immunoassay* **16**, 124–130.
14. Ebner, C., Birkner, T., Valenta, R. *et al.* (1991) Common epitopes of birch pollen and apples – studies by western and northern blot. *J Allergy Clin Immunol* **88**, 588–594.
15. Ebner, C., Hirschwehr, R., Bauer, L. *et al.* (1995) Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* **95**, 962–969.
16. Breiteneder, H., Ferreira, F., Hoffmann-Sommergruber, K. *et al.* (1993) Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur J Biochem* **212**, 355–362.
17. Breiteneder, H., Ferreira, F., Reikerstorfer, A. *et al.* (1992) Complementary DNA cloning and expression in Escherichia coli of Aln g I, the major allergen in pollen of alder (Alnus glutinosa). *J Allergy Clin Immunol* **90**, 909–917.
18. Larsen, N., Stroman, P. & Ipsen, H. (1992) PCR based cloning and sequencing of isogenes encoding the tree pollen major allergen Car b 1 from Carpinus betulus, hornbeam. *Mol Immunol* **29**, 703–711.
19. Kos, T., Hoffmann-Sommergruber, K., Ferreira, F. *et al.* (1993) Purification, characterization and *N*-terminal amino acid sequence of a new major allergen from European chestnut pollen – Cas s 1. *Biochem Biophys Res Commun* **196**, 1086–1092.
20. Hoffmann-Sommergruber, K., Vanek-Krebitz, M., Radauer, C. *et al.* (1997) Genomic characterization of members of the Bet v 1 family: genes coding for allergens and pathogenesis-related proteins share intron positions. *Gene* **197**, 91–100.
21. Jarolim, E., Tejkl, M., Rohac, M. *et al.* (1989) Monoclonal antibodies against birch pollen allergens: characterization by immunoblotting and use for single-step affinity purification of the major allergen Bet v 1. *Int Arch Allergy Appl Immunol* **90**, 54–60.
22. Rohac, M., Birkner, T., Reimitzer, I. *et al.* (1991) The immunological relationship of epitopes on major tree pollen allergens. *Mol Immunol* **28**, 897–906.
23. Swoboda, I., Jilek, A., Ferreira, F. *et al.* (1995) Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* **270**, 2607–2613.
24. Swoboda, I., Scheiner, O., Kraft, D., Breitenbach, M., Heberle-Bors, E. & Vicente, O. (1994) A birch gene family encoding pollen allergens and pathogenesis-related proteins. *Biochim Biophys Acta* **1219**, 457–464.
25. Swoboda, I., Scheiner, O., Heberle Bors, E. & Vicente, O. (1995) CDNA cloning and characterization of three genes in the Bet v 1 gene family that encode pathogenesis-related proteins. *Plant Cell Environ* **18**, 865–874.
26. Korhonen, M., Utriainen, M., Pellinen, R., Valjakka, E., Vapaavuori, E. & Kangasjarvi, J. (1998) The birch (Betula pendula) PR-10 genes are clustered in birch genome and are induced during oxidative stress, leaf sensecence and by pathogens. *Proc of the 5th Int Workshop on Pathgoenesis-related Proteins in Plants Signalling Pathways and Biological Activities*, 9.
27. Son, D.Y., Scheurer, S., Hoffmann, A., Haustein, D. & Vieths, S. (1999) Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *Eur J Nutr* **38**, 201–215.

28. Breiteneder, H., Hoffmann-Sommergruber, K., O'Riordain, G. *et al.* (1995) Molecular characterization of Api g 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17-kDa tree pollen allergens. *Eur J Biochem* **233**, 484–489.
29. Hoffmann-Sommergruber, K., Ferris, R., Pec, M. *et al.* (2000) Characterization of Api g 1.0201, a new member of the Api g 1 family of celery allergens. *Int Arch Allergy Immunol* **122**, 115–123.
30. Wen, J., Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Scheiner, O. & Breiteneder, H. (1997) The potential of Bet v homologous, a nuclear multigene family, as phylogenetic markers in flowering plants. *Mol Phyl Evol* **239**, 281–293.
31. Osmark, P., Boyle, B. & Brisson, N. (1998) Sequential and structural homology between intracellular pathogenesis-related proteins and a group of latex proteins. *Plant Mol Biol* **38**, 1243–1246.
32. Matton, D. & Brisson, N. (1989) Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Mol Plant Microbe Interact* **2**, 325–331.
33. Puehringer, H., Moll, D., Hoffmann Sommergruber, K., Watillon, B., Katinger, H. & da Camara Machado Margit, L. (2000) The promoter of an apple Ypr10 gene, encoding the major allergen Mal d 1, is stress- and pathogen-inducible. *Plant Sci Shannon* **152**, 35–50.
34. Pettenburger, K., Tejkl, M., Valenta, R. *et al.* (1992) Distribution of allergens and allergen-coding mRNAs in various tissues of white birch. *Mol Immunol* **29**, 1401–1406.
35. Itturiaga, E., Leech, M., Barratt, D. & Wang, T. (1994) Two ABA-responsive proteins from pea (Pisum sativum L.) are closely related to intracellular pathogenesis-related proteins. *Plant Mol Biol* **24**, 235–240.
36. Warner, S., Gill, A. & Draper, J. (1994) The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis. *Plant J* **6**, 31–43.
37. Yazaki, K., Bechthold, A. & Tabata, M. (1995) Nucleotide sequence of a cDNA from Lithospermum erythrorhizon homologous to PR-1 of parsley. *Plant Physiol* **108**, 1331–1332.
38. Sikorski, M., Biesiadka, J., Kasperska, A., Kopcinska, J. & Lotocka, B. (1999) Expression of genes encoding PR10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules. *Plant Sci Shannon* **149**, 125–137.
39. Dubos, C. & Plomion, C. (2001) Drought differentially affects expression of a PR-10 protein, in needles of maritime pine (Pinus pinaster Ait.) seedlings. *J Exp Bot* **52**, 1143–1144.
40. Xu, X., Beweley, D. & Greenwood, J. (2000) Cloning and characterization of an 18 kiloDalton protein in the roots of the perennial weed Taraxacum officinale Weber (Dandelion) which has allergen- and pathogenesis-related protein properties. *Plant Cell Environ* **23**, 1227–1236.
41. Utriainen, M., Kokko, H., Auriola, S., Sarrazin, O. & Karenlampi S. (1998) PR-10 protein is induced by copper stress in roots and leaves of a Cu/Zn tolerant clone of birch, Betula pendula. *Plant Cell Environ* **21**, 821–828.
42. Wingate, V., Lawton, M. & Lamb, C. (1988) Glutathione causes a massive and selective induction of plant defense genes. *Plant Physiol* **87**, 206–210.
43. Broderick, K., Pittock, C., Arioli, T., Creaser, E., Weinman, J. & Rolfe, B. (1997) Pathogenesis-related proteins in Trifolium subterraneum: a survey and subsequent characterization of a protein inducible by etephon and redlegg earth mite attack. *Aust J Plant Physiol* **24**, 819–829.
44. Minic, Z., Brown, S., De-Kouchkovsky, Y., Schultze, M. & Staehelin, C. (1998) Purification and characterization of a novel chitinase-lysozyme, of another chitinase, both hydrolysing Rhizobium meliloti Nod factors, and of a pathogenesis-related protein from Medicago sativa roots. *Biochem J* **332**, 329–335.
45. Lo, S., Hipskind, J. & Nicholson, R. (1999) cDNA cloning of a sorghum pathogenesis-related protein (PR10) and differential expression of defense related genes following inoculation with Cochliobolus heterostrophus or Colletotrichum sublineolum. *Mol Plant Microbe Interact* **12**, 479–489.
46. Saraste, M., Sibbald, P.R. & Wittinghofer, A. (1990) The P-loop a common motif in ATP- and GTP-binding proteins. *Trends Biochem Sci* **15**, 430–434.
47. Gajhede, M., Osmark, P., Poulsen, F.M., Ipsen, H., Larsen, J.N., Joost-van-Neerven, R.J., Schou, C., Lowenstein, H. & Spangfort, M.D. (1996) X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol* **3**, 1040–1045.
48. Neudecker, P., Schweimer, K., Nerkamp, J., Scheurer, S., Vieths, S., Sticht, H. & Rosch, P. (2001) Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. *J Biol Chem* **276**, 22756–22763.
49. Moiseyev, G.P., Fedoreyeva, L.I., Zhuravlev, Y.N., Yasnetskaya, E., Jekel, P.A. & Beintema, J.J. (1997) Primary structures of two ribonucleases from ginseng calluses. New members of the PR-10 family of intracellular pathogenesis-related plant proteins. *FEBS Lett* **407**, 207–210.
50. Bufe, A., Spangfort, M.D., Kahlert, H., Schlaak, M. & Becker, W.M. (1996) The major birch pollen allergen, Bet v 1, shows ribonuclease activity. *Planta* **199**, 413–415.

51. Swoboda, I., Hoffmann Sommergruber, K., O'Riordain, G., Scheiner, O., Heberle Bors, E. & Vicente, O. (1996) Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro. *Physiologia Plantarum* **96**, 433–438.
52. Gonneau, M., Pagant, S., Brun, F. & Laloue, M. (2001) Photoaffinity labelling with the cytokinin agonist azido-CPPU of a 34 kDa peptide of the intracellular pethogenesis-related protein family in the moss Physcomitrella patens. *Plant Mol Biol* **46**, 539–548.
53. Bircher, A.J., Van Melle, G., Haller, E., Curty, B. & Frei, P.C. (1994) IgE to food allergens are highly prevalent in patients allergic to pollens, with and without symptoms of food allergy. *Clin Exp Allergy* **24**, 367–374.
54. Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Laimer da Camara Machado, M. *et al*. (1995) Cloning and sequencing of Mal d 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to Bet v 1, the major birch pollen allergen. *Biochem Biophys Res Commun* **214**, 538–551.
55. Scheurer, S., Metzner, K., Haustein, D. & Vieths, S. (1997) Molecular cloning, expression and characterization of Pru a 1, the major cherry allergen. *Mol Immunol* **34**, 619–629.
56. Karamloo, F., Scheurer, S., Wangorsch, A., May, S., Haustein, D. & Vieths, S. (2001) Pyr c 1, the major allergen from pear (*Pyrus communis*), is a new member of the Bet v 1 allergen family. *J Chromatogr B Biomed Sci Appl* **756**, 281–293.
57. Hoffmann-Sommergruber, K., O'Riordain, G., Ahorn, H. *et al*. (1999) Molecular characterization of Dau c 1, the Bet v 1 homologous protein from carrot and its cross-reactivity with Bet v 1 and Api g 1. *Clin Exp Allergy* **29**, 840–847.
58. Jensen-Jarolim, E., Wiedermann, U., Ganglberger, E. *et al*. (1999) Allergen mimotopes in food enhance type I allergic reactions in mice. *Faseb J* **13**, 1586–1592.
59. van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J. *et al*. (2000) Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J Biol Chem* **275**, 11451–14518.
60. Bauer, L., Ebner, C., Hirschwehr, R. *et al*. (1996) IgE cross-reactivity between birch pollen, mugwort pollen and celery is due to at least three distinct cross-reacting allergens: immunoblot investigation of the birch–mugwort–celery syndrome. *Clin Exp Allergy* **26**, 1161–1170.
61. Jankiewicz, A., Baltes, W., Bogl, K. *et al*. (1997) Influence of food processing on the immunochemical stability of celery allergens. *J Sci Food Agric* **75**, 359–370.
62. Hoffmann-Sommergruber, K., Demoly, P., Crameri, R. *et al*. (1999) IgE reactivity to Api g 1, a major celery allergen, in a Central European population is based on primary sensitization by Bet v 1. *J Allergy Clin Immunol* **104**, 478–484.
63. Moneo, I., Gomez, M., Sanchez-Monge, R. *et al*. (1999) Lack of crossreaction with Bet v 1 in patients sensitized to Dau c 1, a carrot allergen. *Ann Allergy Asthma Immunol* **83**, 71–75.
64. Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Laimer-da-Camara-Machado, M. *et al*. (1995) Cloning and sequencing of Mal d 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to Bet v 1, the major birch pollen allergen. *Biochem Biophys Res Commun* **214**, 538–551.
65. Vieths, S. & Schoning, B. (1996) Characterization of Mal d 1, the 18-kD major apple allergen, at the molecular level. *Monogr Allergy* **32**, 63–72.
66. Atkinson, R., Perry, J., Matsui, T., Ross, S. & Macrae, E. (1996) A stress-, pathogenesis-, and allergen-related cDNA in apple fruit is also ripening-related. *NZ J Crop Horticult Sci* **24**, 103–107.
67. Pauli, G., Oster, J.P., Deviller, P. *et al*. (1996) Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: diagnostic value for birch pollen and associated allergies. *J Allergy Clin Immunol* **97**, 1100–1109.
68. Vieths, S., Jankiewicz, A., Schoning, B. & Aulepp, H. (1994) Apple allergy: the IgE-binding potency of apple strains is related to the occurrence of the 18-kDa allergen. *Allergy* **49**, 262–271.
69. Ferreira, F., Hirtenlehner, K., Jilek, A. *et al*. (1996) Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* **183**, 599–609.
70. Ferreira, F., Hebenstreit, D., Kramer, B. *et al*. (2000) Amino Acid Positions involved in the Formation of IgE-binding Epitopes of Api g 1 and Mal d 1 Allergens. *J Allergy Clin Immunol* **105**, S137.
71. Hsieh, L.S., Moos, M. & Lin, Y. (1995) Characterization of apple 18 and 31 kd allergens by micro-sequencing and evaluation of their content during storage and ripening. *J Allergy Clin Immunol* **96**, 960–970.
72. Fritsch, R., Bohle, B., Vollmann, U. *et al*. (1998) Bet v 1, the major birch pollen allergen, and Mal d 1, the major apple allergen, cross-react at the level of allergen-specific T helper cells. *J Allergy Clin Immunol* **102**, 679–686.

73. Bohle, B., Schwihla, H., Hu, H.Z. *et al*. (1998) Long-lived Th2 clones specific for seasonal and perennial allergens can be detected in blood and skin by their TCR-hypervariable regions. *J Immunol* **160**, 2022–2027.
74. Pastorello, E.A., Pravettoni, V., Farioli, L. *et al*. (1999) Clinical role of a lipid transfer protein that acts as a new apple-specific allergen. *J Allergy Clin Immunol* **104**, 1099–1106.
75. Fernandez-Rivas, M., van Ree, R. & Cuevas, M. (1997) Allergy to Rosaceae fruits without related pollinosis. *J Allergy Clin Immunol* **100**, 728–733.
76. Etesamifar, M. & Wuthrich, B. (1998) IgE-vermittelte Nahrungsmittelallergie bei 383 Patienten unter Berücksichtigung des oralen Allergie-Syndroms. *Allergologie* **21**, 451–457.
77. Ballmer-Weber, B.K., Wuthrich, B., Wangorsch, A., Fotisch, K., Altmann, F. & Vieths, S. (2001) Carrot allergy: double-blinded, placebo-controlled food challenge and identification of allergens. *J Allergy Clin Immunol* **108**, 301–307.
78. Jensen-Jarolim, E., Leitner, A., Hirschwehr, R. *et al*. (1997) Characterization of allergens in Apiaceae spices: anise, fennel, coriander and cumin. *Clin Exp Allergy* **27**, 1299–1306.
79. Godnic-Cvar, J., Susani, M., Breiteneder, H. *et al*. (1997) Recombinant Bet v 1, the major birch pollen allergen, induces hypersensitivity reactions equal to those induced by natural Bet v 1 in the airways of patients allergic to tree pollen. *J Allergy Clin Immunol* **99**, 354–359.
80. Bousquet, J., Lockey, R. & Malling, H.J. (1998) Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* **102**, 558–562.
81. Valenta, R., Vrtala, S., Laffer, S., Spitzauer, S. & Kraft, D. (1998) Recombinant allergens. *Allergy* **53**, 552–561.
82. Ferreira, F., Ebner, C., Kramer, B. *et al*. (1998) Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *Faseb J* **12**, 231–242.
83. Vrtala, S., Hirtenlehner, K., Vangelista, L. *et al*. (1997) Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J Clin Invest* **99**, 1673–1681.
84. Pauli, G., Purohit, A., Oster, J.P. *et al*. (2000) Comparison of genetically engineered hypoallergenic rBet v 1 derivatives with rBet v 1 wild-type by skin prick and intradermal testing: results obtained in a French population. *Clin Exp Allergy* **30**, 1076–1084.
85. Vrtala, S., Akdis, C.A., Budak, F. *et al*. (2000) T-cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. *J Immunol* **165**, 6653–6659.
86. Ganglberger, E., Grunberger, K., Wiedermann, U. *et al*. (2001) IgE mimotopes of birch pollen allergen Bet v 1 induce blocking IgG in mice. *Int Arch Allergy Immunol* **124**, 395–397.
87. Jensen-Jarolim, E., Leitner, A., Kalchhauser, H. *et al*. (1998) Peptide mimotopes displayed by phage inhibit antibody binding to bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. *Faseb J* **12**, 1635–1642.
88. Guex, N. & Peitsch, M. (1997) SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis* **18**, 2714–2723.

# 9 Plant Seed Globulin Allergens

E.N. CLARE MILLS, JOHN A. JENKINS and GARY A. BANNON

## 9.1 Introduction

The salt-soluble storage proteins of plant seeds, also known as globulins, were amongst the first proteins to be studied and were described in detail by T.B. Osborne in his classic work published in 1924. In it he describes the sequential extraction of proteins from seeds, now known as *Osborne fractionation*, which combines a series of different solvents, including water, dilute salt solution and aqueous alcohols [1]. Following the extraction of the albumin fraction with water, the globulin fraction is extracted with dilute saline (0.5–1.0 M NaCl). However, we now know that this fractionation does not necessarily give well-defined protein fractions at a molecular level. Thus in certain plant species, such as soybean, the globulins are soluble in lower concentrations of salt (~0.2 M), whilst those from seeds such as Brazil nut and sesame require salt concentrations of around 2 M for solubility.

Globulins are found in a wide range of monocotyledonous and dicotyledonous plant species, with homologues also being found in gymnosperms (conifers) and spores of ferns. In flowering plants, they function primarily as seed storage proteins and are highly abundant comprising up to 50% of the total seed protein in some plant species. Early in the twentieth century these proteins were further characterised on the basis of their sedimentation coefficients ($S_{20w}$) using the analytical ultracentrifuge, and were found to comprise a larger, more slowly sedimenting fraction, with coefficients between 11S and 12S and a smaller fraction, with coefficients between 7S and 8S, a broad classification which is still widely used today.

Originally termed legumins because of their widespread occurrence in legume seeds, the 11/12S storage globulins are multimeric proteins with $M_r \sim 300$–$450\,000$ (Fig. 9.1(A)). In general, they exist as a salt-dependent equilibrium of trimers and hexamers comprising $M_r \sim 50$–$60\,000$ subunits held together by non-covalent interactions. The subunits are the product of a multigene family, one of the first to be characterised in detail being that of soybean where around five genes have been identified [2]. They are synthesised as single polypeptides, which are then post-translationally cleaved to give rise to an acidic ($M_r \sim 30$–$40\,000$) and a basic ($M_r \sim 20\,000$) polypeptide chain linked by a single intermolecular disulphide bond.

The 7/8S globulins also occur in a range of monocotyledonous and dicotyledonous plant species and are often called vicilins because of their presence in the Viciae group of legumes (Fig. 9.1(B)). They are typically trimeric proteins of $M_r$ about $150\,000$–$190\,000$, with subunit $M_r$ ranging from about $40\,000$–$80\,000$ but typically about $50\,000$. Furthermore, proteolytic processing and glycosylation may occur, resulting in a diverse spectrum of components when separated by SDS-PAGE. Vicilins contain no disulphide bonds and consequently, the spectrum of components revealed by SDS-PAGE is similar in the absence or presence of reducing agents.

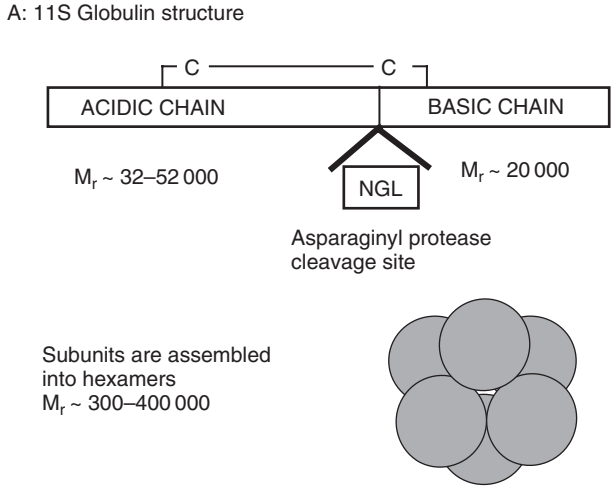


**Fig. 9.1(A)** Schematic diagram illustrating the subunit structure and processing of the 11S.

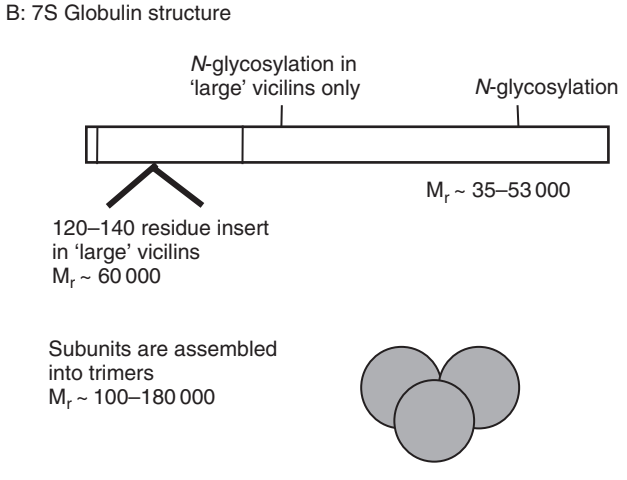


**Fig. 9.1(B)** Schematic diagram illustrating the subunit structure and processing of the 7S seed globulins.

The globulins are synthesised on the rough endoplasmic reticulum (ER) before being transported to a vacuole where they are deposited to form protein bodies. Studies on legumes have shown that the individual 11S and 7S globulin gene sequences are co-ordinately expressed during seed development, with mRNA appearing 35 days after flowering (DAF), reaching a maximum at 70–87 DAF and then decaying to undetectable levels in the mature seed of soybean [2]. In some plant species, such as pumpkin, the packing of the globulins in the protein bodies is highly ordered, adopting a semi-crystalline form. In some cereals, both globulins and prolamin-type storage proteins are deposited in the seed. In oats the prolamins form inclusions within the

globulin protein bodies [3], whilst in rice the globulins are deposited in separate protein bodies to the prolamins.

## 9.2 Globulins – two domain members of the cupin superfamily

Simple sequence comparisons of the globulins have shown that they share only a limited degree of homology, of around 35–45%, which belies their high degree of structural similarity. Three-dimensional structures of one 11S globulin precursor, proglycinin from soybean [4] and three 7S globulins, canavalin [5, 6] from jack bean, phaseolin from French bean [7] and the β-subunit of β-conglycinin from soybean [8] have been determined. These show that both 11S and 7S globulins possess two structurally equivalent *N*-terminal and *C*-terminal domains. Each domain comprises a β-barrel composed of anti-parallel β-strands followed by a number of α-helices, forming a so-called jelly-roll structure, illustrated in Plate 9.1(a) by the trimeric 11S globulin precursor from soybean, proglycinin a(1a)B(1b). Whilst very similar to the three-dimensional structure of the 7S globulins, the latter contains only a few disordered regions comprising ~10% in phaseolin, 12% in β-conglycinin and ~18% in canavlain whilst they represent approximately 20% of the structure in the 11S proglycinin. These mobile regions are likely to protrude from the trimer–trimer interface in the intact hexamer, and may form so-called *entropic bristles*, structures which would be mobile and negatively charged, being rich in amino acid residues such as glutamate. Within the jelly-roll structure, around 30 residues are conserved or conservatively exchanged across the 11S and 7S globulin families [7]. Most of these conserved residues are involved in inter-monomer packing or lie in inter-strand loops, which are presumed to be under some form of constraint (Plate 9.1(b)).

This structural similarity between 11S and 7S globulins was first identified in 1985 [9] and led to the view that they had evolved from a common ancestor [10]. Subsequently related proteins were identified based on patterns of highly conserved residues within the β-barrel structure. These include the sucrose-binding proteins, germin-like proteins and fungal spherulins [11–13]. This superfamily was subsequently termed the *cupins*, based on the Latin name for a small cask or barrel because of their shared β-barrel structure [14, 15]. Thus, the cupins may contain either one or two copies of this structure, the latter proteins being termed bicupins. The best characterised example of a single domain cupin is germin, a protein initially identified as being synthesised during the early stages of wheat embryo germination but now known to exhibit enzymatic activity as oxalate oxidase [16].

## 9.3 Post-translational modification

### 9.3.1 *Glycosylation*

The 11S globulins are rarely glycosylated, but one example is the major $M_r$ 44 000 acidic subunit of the lupin globulin [17]. The presence of a serine residue in a conserved region towards the *N*-terminus of the acidic subunit of this protein indicated

the presence of an *N*-glycosylation consensus sequence (i.e. N–X–S) that is absent from the acidic polypeptides of other 11S globulins. This single point mutation results in glycosylation with about 1% (w/w) carbohydrate being attached [18] which appears to affect subsequent folding, assembly and proteolytic processing. In contrast, the 7S globulins are frequently glycosylated with between one and two *N*-linked glycosylation sites being located in the *C*-terminal domains of the proteins. In general the glycosylation of secreted proteins, such as the globulins involves addition of the oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, to asparagine residues within the consensus glycosylation sites as the polypeptide chain enters the lumen of the ER. This core glycan is then modified during subsequent transport of the protein to the vacuole, via the Golgi apparatus with trimming of the glucose being a key event in the assembly of monomers into trimers [19]. In the case of the 7S globulin of peanut, conarachin, this results in a heterogeneous mixture of *N*-glycans including $Man_{5-6}GlcNAc_2$ and $Man_{3-4}XylGlcNAc_2$ [20]. 7S globulins possessing more than one glycosylation site can exist in several forms. For example, phaseolin, the 7S globulin of French bean, has glycosylation sites at Asn 228 and 317. A species that is singly glycosylated at Asn 228 has been identified, together with a species doubly glycosylated at both asparagine residues [21].

### 9.3.2 *Polyamine conjugation*

It has recently been shown that the 11S globulin of soybean, glycinin, is conjugated to polyamines such as putrescine, in the seed [22]. It is thought that the polyamines are covalently attached to the seed globulin through a γ-glutamyl bond and may have a role in signalling the rapid degradation of the globulins following imbibition of the seed.

### 9.3.3 *Proteolysis*

Both 11S and 7S globulins may undergo proteolytic processing within the seed to yield the mature protein. This occurs in all 11S globulins which, on being deposited in the protein bodies, are cleaved to yield the $M_r$ 30–40 000 acidic and the $M_r$ 20 000 basic polypeptides characteristic of the mature protein. After initial assembly in the ER as trimers, this proteolytic event triggers the subsequent assembly into hexamers (Fig. 9.2). The cleavage site is conserved in 11S globulins across a wide range of plant species and is characteristically comprised of asparagine and glycine residues lying in a mobile loop on the surface of the protein [4]. It appears that this cleavage is performed by a specific asparaginyl endopeptidase, which has an absolute specificity for Asn on the *N*-terminal side of the cleavage site, but has little specificity for amino acids on the *C*-terminal side and is an absolute requirement for subsequent assembly of the hexamers [23]. Proteolytic processing in the seed also occurs to a number of 7S globulins, notably those from pea and lentil to yield a series of polypeptides of $M_r$~12–50 000, including the intact protein [24, 25]. Despite such processing, the 7S globulins are held together by non-covalent forces to retain the intact $M_r$~150 000 trimeric globulin.

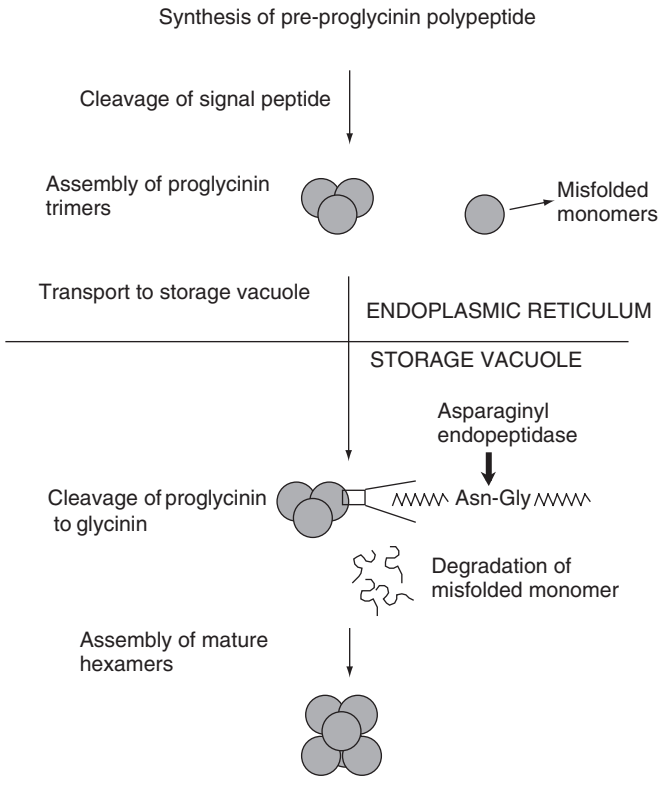


**Fig. 9.2** Schematic diagram illustrating the synthesis and postranslational processing of the 11S seed globulin.

## 9.4 Seed globulin allergens

As a consequence of their presence in many edible plant seeds, the globulins make a significant contribution to the human diet. This is illustrated in Fig. 9.3, which shows an SDS-PAGE separation of total protein extracts from a number of legume species, whilst Table 9.1 lists many of the plant seeds that contain appreciable proportions of 11S and/or 7S globulins. Globulins from soybean are probably amongst the most widely consumed, with around 0.6 million metric tonnes of soybean protein being destined for human consumption in the USA alone [26]. Other species, such as peanut, various other legumes and hazelnut also make significant contributions to the human diet, in contrast to seeds such as sunflower, sesame and poppy, which tend to be consumed in much smaller amounts in Europe and North America, as toppings and garnishes. However, consumption of sesame in particular is more widespread in southern Europe and the Middle East in foods such as tahini. Globulins from peanut, soybean, walnut, lentil, almond and cashew nut have been demonstrated to be allergens and the remainder of this review will focus on these.

**Table 9.1** 7S and 11S globulins in food

| Plant food species | 11S Globulins | 7S Globulins | Allergenicity |
|---|---|---|---|
| **Legumes** | | | |
| Soy bean (*Glycine max*) | Glycinin<br>Acidic $M_r$ ~ 32 000<br>Basic $M_r$ ~ 20 000<br>Approximately five genes code for glycinin [2]<br>The structure of one isoform, proglycinin A1aB1b has been defined [4] | β-Conglycinin<br>α,α′ subunits $M_r$ ~ 60 000;<br>β subunit $M_r$ ~ 50 000<br>The structure of the β subunit has been defined [78] | Both globulins are thought to be allergens; IgE epitopes are located in the acidic subunit of glycinin [35, 44, 45, 76] |
| Pea (*Pisum sativum*) | Legumin<br>Acidic $M_r$ ~ 45 000<br>Basic $M_r$ ~ 20–25 000 [25] | Vicilin<br>$M_r$ ~ 75, 56, 43, 33, 25, 12 000 [24, 25] | Not known; allergy to green peas is uncommon |
| Bean (*Phaseolus vulgaris*) | Minor component | Phaseolin<br>α subunits $M_r$ ~ 51–53 000<br>β subunits $M_r$ ~ 47–48 000<br>γ subunits $M_r$ ~ 43–46 000<br>Contains ~ 35% carbohydrate which is largely responsible for the subunit heterogeneity [77]<br>The β subunits dominate the crystals used to determine its three-dimensional structure [7] | Not known; allergy to beans is uncommon |
| Peanut (*Arachis hypogea*) | Arachin (Ara h 3, Ara h 4)<br>Acidic $M_r$ ~ 40 000<br>Basic $M_r$ ~ 20 000 [78] | Conarachin (Ara h 1)<br>$M_r$ ~ 60 000<br>*N*-glycosylated [20, 31] | Major peanut allergen with epitopes identified in 11S and 7S globulins [31, 33, 38, 39, 74] |
| Lentils (*Lens culinaris*) | Acidic $M_r$ ~ 32–40 000<br>Basic $M_r$ ~ 20 000 | $M_r$ ~ 35–55 000 and 60 000 | Allergenic fragments have been identified for the 7S globulin [48] |
| Chickpea (*Cicer arietunum*) | Legumin<br>Acidic $M_r$ ~ 32–40 000<br>Basic $M_r$ ~ 20 000<br>Main storage protein of Chickpea [79] | $M_r$ ~ 60 000 | Not known as allergens although allergy to chickpea has been reported [80] |

| | | | |
|---|---|---|---|
| Lupin (*Lupinus albus*) | Conglutin-α<br>Acidic $M_r$ ~ 44–52 000<br>Basic $M_r$ ~ 21 000<br>A typical 11S globulin as it is *N*-glycosylated | Conglutin-β<br>$M_r$ ~ 20–60 000 [81] | Not known as allergens although allergy to lupin has been reported |
| **Other plant species** | | | |
| Coconut (*Cocosin nucifera*) | Cocosin<br>Acidic $M_r$ ~ 32–35 000<br>Basic $M_r$ ~ 22 000 [82] | Not characterised | Cross-reactive allergens related to walnut globulin allergens have been identified [50] |
| Walnut (*Juglans regia*) | Acidic $M_r$ ~ 32 000<br>Basic $M_r$ ~ 22 000 | Jug r 2<br>$M_r$ ~ 47 000 | The 7S globulin has been identified as an allergen [47] |
| Almond (*Prunus amygdalus*) | Amandin, Major Almond Protein (MAP)<br>Acidic $M_r$ ~ 38–42 000<br>Basic $M_r$ ~ 20–22 000 [83] | Not characterised | Data indicate that amandin is an allergen [51, 82] |
| Cashewnut (*Anacardium occidental*) | Ancardium (13S)<br>Acidic $M_r$ ~ 30–37 000<br>Basic $M_r$ ~ 18–24 000 [91] | Ana o 1<br>$M_r$ ~ 50 000 | Both 11S and 7S globulins have been identified as allergens [49] |
| Brazil nut (*Bertholetica excelsis*) | Excelsin<br>Acidic $M_r$ ~ 51–29 000<br>Basic $M_r$ ~ 11–17 000 [84] | Minor component | Not known as an allergen although Brazil nut is an allergenic tree nut [85] |
| Sesame (*Sesamum indicum*) | α-Globulin<br>Acidic $M_r$ ~ 48–51 500<br>Basic $M_r$ ~ 28 800–31 000 [86, 87, 88] | Minor component | Preliminary data indicate that the 11S globulin is an allergen [49] |
| Pumpkin (*Cucurbita moschata*) | Cucurbitin<br>Acidic $M_r$ ~ 30–36 000<br>Basic $M_r$ ~ 21–25 000 [89] | Not described | Not known; allergy to pumpkin seeds has been reported [90] |

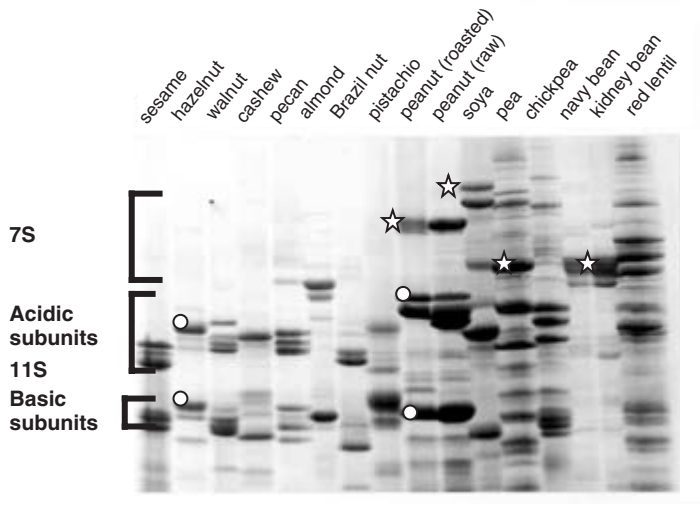


**Fig. 9.3** Reducing SDS-PAGE analysis of total protein extracts from a range of legume species. Closed circles mark the 11S globulin subunits; stars mark the 7S globulin subunits.

### 9.4.1 *Allergenic seed globulins*

#### 9.4.1.1 *Peanut globulin allergens*

9.4.1.1.1 *Peanut varieties.* The peanut (*Arachis hypogaea* L.), also known as groundnut, is an annual plant belonging to the family Leguminosae (legumes) and is native to South America. Several peanut varieties are grown in the United States, including Virginia, Spanish and runners. Various studies over the last several years have examined the nature and location of the multiple allergens in peanuts [27, 28]. These studies showed the allergenic component of the peanut was in the protein of the cotyledon and did not differ significantly between the different varieties of peanuts [29].

9.4.1.1.2 *Classification and biochemical characterisation of peanut allergens.* Numerous peanut proteins have been identified as allergens by their ability to bind IgE from peanut-allergic patients. The first major peanut allergen identified in this manner was Ara h 1 [30]. The Ara h 1 protein has an $M_r$ of 63 500 on SDS-PAGE gels and an isoelectric point of 4.55. The gene for this allergen has recently been cloned and sequenced and found to have significant sequence homology with the plant 7S globulin proteins [31]. Ara h 1 is known to be glycosylated and has one asparagine consensus carbohydrate addition site [32, 20]. More than 90% of patients with positive challenges to peanut have specific IgE to Ara h 1 and based on IgE recognition, it is considered one of the major allergens of peanut. A third peanut allergen (Ara h 3) was identified by using soybean-adsorbed serum IgE from peanut-allergic patients [32], an approach used to identify proteins specific to peanut allergy. As expected, both

Ara h1 and Ara h 2 (the allergenic 2S albumin of peanut, see Chapter 3) were identified by this process, together with a third protein. The deduced amino acid sequence of this protein, named Ara h 3, was found to be homologous to the 11S family of seed storage proteins. The recombinant form of this allergen was expressed in a bacterial system and was recognised by serum IgE from ~50% of a peanut-allergic patient population [33]. Four other proteins have been identified as peanut allergens and designated Ara h 4–7 [34]. With the exception of Ara h 5, they all share significant homology with either Ara h 1, 2 or 3 [34, 35]. Ara h 5 is a member of the profilin family but is only recognised by IgE from a small fraction (13%) of the peanut-allergic population [34].

In summary, two of the major peanut allergens identified thus far belong to the abundant globulin seed storage protein families (7S and 11S) and together with the other major peanut allergen, which belongs to the 2S albumin storage family, comprise a large percentage of the total protein found in the peanut seed.

9.4.1.1.3 *Mapping and characterisation of peanut allergen IgE-binding epitopes.* Two categories of IgE-binding epitopes, linear and conformational, are generally accepted to occur in food allergens. Conformational epitopes occur when the secondary or tertiary structure of the allergen brings together different segments of the polypeptide chain to form the IgE-binding site. In contrast, linear epitopes only require the primary amino acid sequence of the allergen for IgE to bind. While conformational IgE-binding epitopes are prevalent and important to the aetiology of aeroallergen-mediated allergic reactions, linear epitopes are important for food allergens mainly because the immune system will encounter such allergens only after they have been partially denatured and digested by the human gastrointestinal (GI) tract. Therefore, the linear IgE-binding epitopes of food allergens have attracted more attention than the less prevalent conformational epitopes.

Overlapping peptides and serum IgE from patients with documented peanut hypersensitivity were used to identify the linear IgE-binding epitopes on Ara h 1 and Ara h 3. At least 23 different IgE-binding epitopes have been identified located throughout the length of the Ara h 1 molecule [36], together with four IgE-binding epitopes on Ara h 3 [37, 38]. All of the epitopes were 6–15 amino acids in length, but no obvious sequence motif was shared by all peptides. Four of the Ara h 1 epitopes appeared to be immunodominant IgE-binding peptides in that they were recognised by serum from more than 80% of the patients tested and bound more IgE than any of the other Ara h 1 IgE-binding epitopes. Similarly, one of the Ara h 3 epitopes was also determined to be immunodominant.

Mutational analysis of each of the IgE-binding epitopes from the peanut allergens revealed that single amino acid changes within these peptides had dramatic effects on IgE-binding characteristics. Surprisingly, substitution of a single amino acid led to the loss of IgE binding [37–39]. Analysis of the type and position of amino acids within the IgE-binding epitopes indicated that substitution of hydrophobic residues in the centre of the epitopes was more likely to lead to a loss of IgE binding than substitutions at the *N*- and *C*-terminal ends [39]. These results represent the first systematic analysis of the IgE-binding epitopes of food allergens and, as such, will be important to be taken into consideration when developing strategies for the treatment and prevention of peanut allergy.

9.4.1.2 *Soybean globulin allergens.* Soybean proteins are widely used in processed food products, mainly because of their cost, functional properties, and to more limited extent their nutritional properties [40]. New processing methods have created a generation of soybean protein isolates with mild flavours and aromas, as well as improved functionality. Soybean proteins can be incorporated into a variety of food products at levels high enough to have an effect on the health of soybean-sensitive individuals. This has driven the food industry and soybean chemists to define the protein components responsible for soybean allergenicity. Soybeans belong to the legume family of plants that also includes peanuts. Similar to the case with peanuts, multiple allergens have also been identified in soybean including glycinin, β-conglycinin and P34 [36, 41–45].

Of the soybean allergens, P34 and G2 glycinin have been the most extensively studied with regard to their allergenicity. P34 is a cysteine protease and the most commonly recognised allergen, being recognised by serum IgE from 65% of soybean-allergic patients [42]. In a manner similar to that used for peanut allergens, linear IgE-binding epitopes have been identified using synthetic overlapping peptides and sera from soybean-allergic patients. For the P34 allergen, 16 linear IgE-binding epitopes were identified, nine of which were mapped to the mature protein [43]. Eleven epitopes were identified on the G2 glycinin protein, four of which mapped to the basic chain while seven mapped to the acidic chain [46]. All of the epitopes were 10–15 amino acids in length, but there was no obvious sequence motif shared by all peptides. Five of the epitopes on the P34 allergen and four of the epitopes on the G2 glycinin allergen were identified as immunodominant by their ability to bind IgE from a majority of soybean-allergic patients tested. The disposition of the epitopes on the proglycinin structure defined by Adachi and co-workers [4] is shown in Plate 9.2.

In contrast to the peanut allergens, mutational analysis of each of the IgE-binding epitopes from the soybean G2 glycinin revealed that single amino acid changes within these peptides did not always have a dramatic effect on IgE-binding characteristics. Only one of the IgE-binding epitopes could be rendered non-IgE binding by alanine substitutions in the peptide [46]. Whether this represents a distinction between the immune system's response to soybean and peanut allergens or some other difference inherent in soybean and peanut allergy has yet to be determined.

9.4.1.3 *Other allergenic globulins.* Whilst the globulins of peanut and soybean are the best characterised globulin food allergens, other members of this family have been implicated as food allergens. These include the 7S globulin of walnut (Jug r 2) [47], one of the subunits of the proteolytically processed 7S globulin of lentils [48] and the 7S globulins of sesame [49] and cashew nut [50] together with the 11S globulins of coconut, walnut [51] and almond (also known as almond major protein, AMP) [52].

## 9.5 Effect of thermal processing on allergenic globulins

Whilst soybean and lentil allergens are almost always consumed following cooking, those from nuts such as walnuts, are often eaten raw, whilst peanuts are often subjected to roasting, a thermal treatment performed at low levels of hydration, a factor which may alter the way in which the proteins are denatured and aggregate. The globulin

storage proteins all share a propensity to form large thermally induced aggregates which form the basis of the widespread utilisation of soybean proteins in foods as it can form heat-set gel networks [53]. As a consequence of their importance in our diet, most studies have been devoted to soybean globulins. Thus the 11S globulin of soybean, glycinin, will form a cryoprecipitate on cooling to 4°C, at concentrations >1%, and will aggregate on heating, forming heat-set gels at around 2.5–10%, depending on pH and ionic strength. Both 11S and 7S globulins, in common with other members of the cupin superfamily, exhibit considerable thermal stability, with 7S globulins having their major thermal transition at around 70–75°C, whilst 11S globulins unfold at temperatures above 94°C, as determined by differential scanning calorimetry, the precise values varying between plant species, protein concentration and ionic strength. For the 11S globulins, heating for extended periods at 100°C apparently results in almost complete unfolding, as determined by circular dichroism (CD) spectroscopy, and is accompanied by dissociation of the acidic and basic polypeptides resulting from cleavage of the connecting disulphide bond and aggregation [53, 54]. Similar large thermally induced aggregates form on heating the 7S globulin of soybean, β-conglycinin [55].

Of the other allergenic globulins, only Ara h1, the 7S globulin of peanut, has been studied in any depth [56]. These workers found that, like many other 7S globulins, Ara h 1 is relatively thermostable, undergoing an irreversible co-operative transition with a maximum at 87°C, when heated in solution. This is accompanied by unfolding and aggregation of the protein which forms stable dimers, trimers and larger structures. However, Ara h 1 purified from residual soluble protein from roasted nuts behaved differently. Thus, when heated *in situ* in the nut, Ara h 1 only became unfolded on heating to 140 °C for 15 minutes. Such increases in thermostability of proteins are often encountered in low-water systems, such as whole food matrices [57]. Both the residual native and denatured insouble protein retained their IgE reactivity, implying that either the native protein contains many thermostable epitopes or that individuals develop IgE responses primarily towards short, continuous epitopes found in the cooked material.

In addition to the effect of thermal processing on the three-dimensional structure and aggregation state of globulins, the proteins probably undergo covalent modification as a consequence of chemical reactions that occur in foods on heating. Many of these reactions are important in developing the flavour and aroma characteristics of foods and frequently involve glycation and subsequent Maillard rearrangements between amino groups on the proteins and sugars present in foods. Thus, the allergenic activity of the peanut 7S globulin allergen, Ara h1, was found to increase following deliberate Maillard modification *in vitro*, both in terms of IgE binding and resistance to digestion in addition to increasing its thermostability. This reflected the dramatic increase in allergenic activity of peanut extracts from roasted, compared with raw nuts [58]. Indeed it is now apparent that certain methods of cooking peanuts, such as boiling or frying, are more effective at reducing allergenicity, compared with dry roasting [59].

## 9.6 Effect of digestion on allergenic globulins

There is a great deal of indirect information on the digestibility of globulins in general, and specifically the allergenic globulins from soybean, from studies on the

protein utilisation of legumes, particularly in relation to animal husbandry. In general, seed globulins are less well digested than other protein sources such as cow's milk, even when legumes are processed to remove the activity of anti-nutritional factors such as trypsin inhibitors and lectins. Thus, rat studies employing purified legume globulins showed that around 40% of the protein remained in the small intestine after 1 hour [60]. A study of globulin digestion in calves fed soya flour has also shown that immunoreactive globulin fragments appear in the ileum 4–6 hours after feeding [61]. Other studies have shown that the degree of digestion of legume proteins is dependent on the effect of processing on the globulins themselves [60, 61–63]. It should also be noted that soybean globulins are partially or fully insoluble between pH 3.5 and 6.5 and therefore only limited solubilisation of globulins would occur when they enter the stomach.

Both glycinin and β-conglycinin are susceptible to proteolysis by pepsin [64] and both the 11S globulins of soybean (glycinin) and pea have been shown to form stable intermediates of $M_r \sim 280\,000$ (known as glycinin T and legumin T respectively) on trypsinolysis, similar intermediates also being formed following chymotrypsinolysis. Despite clipping of the acidic subunits by partial trypsinolysis to form $M_r$ 13 000 and 16 000 fragments, the basic subunits remain intact, the resulting intermediates retaining the quaternary structure characteristic of 11S globulins [65, 66]. Studies using a monoclonal antibody that preferentially recognised these proteolytic intermediates indicate that they may also form during digestion *in vivo* in experimental animals [67]. Such stable intermediates may also result from trypsinolysis of 7S globulins, such as β-conglycinin, with two $M_r$ 31 550 and 29 500 peptides having been shown to originate from the α/α′ subunits and a third $M_r$ 31 500 peptide from the β subunit [68]. Similar sized products were also observed by Shutov *et al.* [12] and may correspond to the *N*- and/or *C*-terminal domains of the α, α′ subunits.

One factor that has been neglected is the impact of other food constituents on the stability properties of allergens. Thus, whilst saponins have been implicated in increasing gut permeability to allergens [69], it is also clear that these compounds interact with soybean globulins, greatly increasing their stability to the enzyme chymotrypsin [70]. Similarly, plant phenolics can form covalent adducts with globulins [71] although it has not been determined whether these are formed during conventional food processing procedures.

## 9.7 Role of globulin structure and properties in allergenicity

### 9.7.1 *Structure and stability*

The cupin fold found in the globulin seed storage family of proteins appears to be a remarkably stable structural motif, a property shared by other members of the cupin superfamily. Indeed procedures for purification of certain globulins (such as phaseolin from beans), developed when protein biochemistry was in its infancy, incorporated a heat treatment to remove unwanted, less stable, proteins [72] whilst the isolation procedure for oxalate oxidase from wheat bran involves digestion with pepsin and boiling for 30 minutes [16]. In many protein families, including many of the allergen

families such as the prolamin superfamily described elsewhere in this book, disulphide bonds make an important contribution to protein stability. However, there are no disulphide bonds in the 7S globulins and relatively few in the 11S globulin structure with, for example, a maximum of three possible in glycinin, one joining the acidic and basic polypeptides, a second in the *N*-terminal domain and two cysteine in the large disordered region which may possibly form a disulphide bridge, although this has yet to be identified.

In addition to disulphide bonds, proteins are stabilised by forces such as the hydrophobic effect, hydrogen bonding and packing (van der Waals) interactions. These interactions make a large favourable contribution to the free energy of folding, which is offset by the large unfavourable entropic effect of folding. In addition, the globulins also have regions, which exhibit a high degree of mobility even in the native structure [4]. The binding of a monoclonal antibody probe, specific for an epitope within a mobile region of glycinin, was unaltered even after heat-induced formation of large aggregates, indicating that at least one mobile region is still present following cooking [54]. Evidence is accumulating that the stability of seed globulins, along with the cupin superfamily in general, relates to the inherent stability of the β-barrel core. Such stability probably plays an important role in allowing sufficient immunologically active fragments to pass down the GI tract.

## 9.8 Role of peanut allergen structure in defining IgE-binding epitopes

One of the important characteristics of food allergens is that they are resistant to degradation during processing and digestion [73]. In order to discover the underlying reasons for this, the structure of one of the peanut allergens, Ara h 1, was studied in relation to its IgE-binding epitopes. Structural analysis was undertaken using a variety of technologies including CD spectroscopy, resistance to digestion, fluorescence anisotropy and computer modelling of protein structure.

Ara h 1 was shown to form a highly stable homotrimer [39] that was stabilised by hydrophobic interactions. A molecular model of the Ara h 1 trimer was constructed to view the stabilising hydrophobic residues in the three-dimensional structure. Hydrophobic amino acids that contribute to trimer formation are at the distal ends of the three-dimensional structure where monomer–monomer contacts occur. Coincidentally, the majority of the IgE-binding epitopes are also located in this region suggesting that they may be protected from digestion by the monomer–monomer contacts. Various protease-resistant fragments containing multiple IgE-binding sites were identified after incubation of Ara h 1 with digestive enzymes, these peptide fragments being protected from digestion for up to 3 hours [74].

## 9.9 Conclusions

It is clear that the storage globulins can act as major food allergens by virtue of their abundance in foods and their high stability. However, whilst characterised as important allergens in peanut and soybean, their role in the allergenicity of other nuts and seeds

where they are abundant components is less clear. An example of this is Brazil nut, where a large proportion of the seed protein is the 11S globulin excelsin, and yet the major allergen in this plant food species is the 2S albumin (see Chapter 3). The low solubility and stability of these proteins at low ionic strength ($I < 0.05$) may have affected the representation of allergens in the dilute salt solutions routinely used to prepare protein fractions for diagnosis. Certainly many seed globulins, such as those from Brazil nuts and sesame seeds, require high salt concentrations to retain their three-dimensional structures and many globulins will disassemble and even denature in low salt or water, forming insoluble aggregates [75]. It may be that these proteins will either be identified as allergens in the future, or that interactions with other proteins may modulate their allergenic properties.

## References


1. Osborne, T.B. (1924) *The Vegetable Proteins*, 2nd edn. Longmans Green and Co., London.
2. Nielsen, N.C., Dickinson, C.D., Cho, T.J. *et al*. (1989) Characterization of the glycinin gene family in soybean. *The Plant Cell* **1**, 313–328.
3. Burgess, S.R. & Miflin, B.J. (1985) The localization of oat (*Avena sativa* L.) seed globulins in protein bodies. *J Experiment Bot* **36**, 945–954.
4. Adachi, M., Takenaka, Y., Gidamis, A.B., Mikami, B. & Utsumi, S. (2001) Crystal structure of soybean proglycinin A1aB1b homotrimer. *J Mol Biol* **12**, 291–305.
5. Ko, T.P., Ng, J.D. & McPherson, A. (1993) The three-dimensional structure of canavalin from jack bean (*Canavalia ensiformis*). *Plant Physiol* **101**, 729–744.
6. Ko, T.P., Day, J. & McPherson, A. (2000) The refined structure of canavalin from jack bean in two crystal forms at 2.1 and 2.0 Å resolution. *Acta Crystallogr D Biol Crystallogr* **56**, 411–420.
7. Lawrence, M.C., Izard, T., Beuchat, M., Blagrove, R.J. & Colman, P.M. (1994) Structure of phaseolin at 2.2 Å resolution. Implications for a common vicilin/legumin structure and the genetic engineering of seed storage proteins. *J Mol Biol* **238**, 748–776.
8. Maruyama, N., Adachi, M., Takahashi, K. *et al.* (2001) Crystal structures of recombinant and native soybean beta-conglycinin beta homotrimers. *Eur J Biochem* **68**, 3595–3604.
9. Argos, P., Narayana, S.V. & Nielsen, N.C. (1985) Structural similarity between legumin and vicilin storage proteins from legumes. *EMBO J* **4**, 1111–1117.
10. Gibbs, P.E., Strongin, K.B. & McPherson, M.A. (1989) Evolution of legume seed storage proteins – a domain common to legumins and vicilins is duplicated in vicilins. *Mol Biol Evol* **6**, 614–623.
11. Shutov, A.D., Kalhovskaya, I.A., Braun, H., Bäumlein, H.B. & Müntz, K. (1995) Legumin-like and vicilin-like seed storage proteins: evidence for a common single-domain ancestral gene. *J Mol Evol* **41**, 1057–1069.
12. Shutov, A.D., Kakhovskaya, I.A., Bastrygina, A.S., Bulmaga, V.P., Horstman, C. & Muntz, K. (1996) Limited proteolysis of β-conglycinin and glycinin, the 7S and 11S storage globulins from soybean (*Glycine max*(L.) Merr.). Structural and evolutionary implications. *Eur J Biochem* **241**, 221–228.
13. Shutov, A.D., Braun, H., Cesnokov, Y.V. & Bäumlein, H.B. (1998) A gene encoding a vicilin-like protein is specifically expressed in fern spores. Evolutionary pathway of seed storage globulins. *Eur J Biochem* **252**, 79–89.
14. Dunwell, J.M. (1998) Cupins: a new superfamily of functionally diverse proteins that include germins and plant storage proteins. *Biotechnol Genet Eng Rev* **15**, 1–32.
15. Dunwell, J.M. & Gane, P.J. (1998) Microbial relatives of seed storage proteins: conservation of motifs in a functionally diverse superfamily of enzymes. *J Mol Evol* **46**, 147–154.
16. Woo, E.J., Dunwell, J.M., Goodenough, P.W., Marvier, A.C. & Pickersgill, R.W. (2000) Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nat Struct Biol* **7**, 1036–1040.
17. Duranti, M., Horstmann, C., Gilroy, J. & Croy, R.R.D. (1995) The molecular basis for *N*-glycosylation in the 11S globulin (legumin) of lupin seed. *J Protein Chem* **14**, 107–110.
18. Duranti, M., Restani, P., Poniatowska, T. & Cerletti, P. (1981) The seed globulins of *Lupinus albus*. *Phytochem* **20**, 2071–2075.

19. Lupatelli, F., Pedrazzini, E., Bollini, R., Vitale, A. & Ceriotti, A. (1997) The rate of phaseolin assembly is controlled by the glucosylation state of its N-linked oligosaccharide chains. *The Plant Cell* **9**, 597–609.
20. van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J. *et al*. (2000) Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J Biol Chem* **275**, 11451–11458.
21. Sturm, A., Van Kuik, J.A., Vliegenthart, J.F. & Chrispeels, M.J. (1987) Structure, position, and biosynthesis of the high mannose and the complex oligosaccharide side chains of the bean storage protein phaseolin. *J Biol Chem* **262**, 13392–13403.
22. Kang, H., Lee, S.G. & Cho, Y.D. (1998) Identification of glycinin *in vivo* as a polyamine-conjugated protein via a γ-glutamyl linkage. *Biochem J* **332**, 467–473.
23. Jung, R., Scott, M.P., Nam, Y.W. *et al*. (1989) The role of proteolysis in the processing and assembly of 11S seed globulins. *The Plant Cell* **10**, 343–357.
24. Gatehouse, J.A., Croy, R.R., Morton, H., Tyler, M. & Boulter, D. (1981) Characterisation and subunit structures of the vicilin storage proteins of pea (*Pisum sativum* L.). *Eur J Biochem* **118**, 627–633.
25. Casey, R., Domoney, C. & Ellis, N. (1986) Legume storage proteins and their genes. *Oxford Surveys of Plant Mol Cell Biol* **3**, 1–95.
26. Hettiarachchy, N. & Kalapathy, U. (1997) Soybean protein products. In: *Soybeans: Chemistry, Technology and Utilisation*, Liu, K. (ed.). Chapman and Hall, New York, pp. 379–411.
27. Bush, R.K., Taylor, S.L. & Nordlee, J.A. (1989) Peanut sensitivity. *Allergy Proc* **10**, 261–264.
28. Gillespie, D.N., Nakajima, S. & Gleich, G.J. (1976) Detection of allergy to nuts by the radioallergosorbent test. *J Allergy Clin Immunol* **57**, 302–309.
29. Taylor, S.L., Busse, W.W., Sachs, M.I., Parker, J.L. & Yunginger, J.W. (1981) Peanut oil is not allergenic to peanut-sensitive individuals. *J Allergy Clin Immunol* **68**, 372–375.
30. Burks, A.W., Williams, L.W., Helm, R.M., Connaughton, C., Cockrell, G. & O'Brien, T. (1991) Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *J Allergy Clin Immunol* **88**, 172–179.
31. Burks, A.W., Cockrell, G., Stanley, J.S., Helm, R.M. & Bannon, G.A. (1995) Recombinant peanut allergen Ara h I expression and IgE binding in patients with peanut hypersensitivity. *J Clin Invest* **96**, 1715–1721.
32. Eigenmann, P.A., Burks, A.W., Bannon, G.A. & Sampson, H.A. (1996) Identification of unique peanut and soy allergens in sera adsorbed with cross-reacting antibodies. *J Allergy Clin Immunol* **98**, 969–978.
33. Rabjohn, P., West, C.M., Connaughton, C. *et al*. (2002) Modification of peanut allergen Ara h 3: Effects on IgE binding and T-cell stimulation. *Int Archives Allergy Immunol* **128**, 15–23.
34. Kleber-Janicke, T., Crameri, R., Appenzeller, U., Schlaak, M. & Becker, W.-M. (1999) Selective cloning of peanut allergens including profilin and 2S albumins, by phage display technology. *Int Archives Allergy Immunol* **119**, 265–274.
35. Bannon, G.A., Kopper, R.A., Maleki, S.M., Shin, D.S., Sampson, H.A. & Burks, A.W. (2000) Structure plays a critical role in the allergenicity of food proteins. *J Allergy Clin Immunol* **105**, 554.
36. Burks, A.W., Brooks, J.R. & Sampson, H.A. (1988) Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *J Allergy Clin Immunol* **81**, 1135–1142.
37. Burks, A.W., Shin, D., Cockrell, G., Stanley, J.S., Helm, R.M. & Bannon, G.A. (1997) Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* **245**, 334–339.
38. Rabjohn, P., Helm, E.M., Stanley, J.S. *et al*. (1999) Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* **103**, 535–542.
39. Shin, D.S., Compadre, C.M., Maleki, S.M. *et al*. (1998) Biochemical and structural analysis of the IgE binding sites on Ara h1, an abundant and highly allergenic peanut protein. *J Biol Chem* **273**, 13753–13759.
40. Anderson, J.A. & Sogn, D.D. (1984) *Adverse Reactions to Foods*. Hyattsville, NIH publication, No. 84-2442.
41. Ogawa, T., Bando, N., Tsuji, H., Okijima, H., Hishikawa, L. & Sasoka, K. (1991) Investigations of the IgE-binding proteins in soybean by immunoblotting with sera from soybean-sensitive patients with atopic dermatitis. *J Nutr Sci Vitamin* **37**, 555–565.
42. Ogawa, F., Tsuji, J., Bando, N. *et al*. (1993) Identification of the soybean allergenic protein, Gly m Bd 30K, with the soybean seed 34-kDa oil-body-associated protein. *Biosci Biotechnol Biochem* **57**, 1030–1033.
43. Helm, R., Cockrell, G., Herman, E., Burks, A.W., Sampson, H.A. & Bannon, G.A. (1998) Cellular and molecular characterization of a major soy allergen. *Int Arch Allergy Immunol* **117**, 29–37.
44. Besler, M. (1999) Soybean (*Glycine Max*). *Internet Symposium on Food Allergens* **1**, 51–79.

45. Helm, R., Cockrell, G., Connaughton, C. *et al*. (2000) A soybean G2 glycinin allergen. 1. Identification and characterization. *Int Arch Allergy Immunol* **123**, 205–212.
46. Helm, R., Cockrell, G., Connaughton, C. *et al*. (2000) A soybean G2 glycinin allergen. 2. Epitope mapping and three-dimensional modelling. *Int Arch Allergy Immunol* **123**, 213–219.
47. Teuber, S., Jarvis, K.C., Dandekar, A.M., Peterson, W.R. & Ansari, A. (1999) Identification and cloning of a complimentary DNA encoding a vicilin-like proprotein, Jug r 2, from English walnut kernel (*Juglans regia*), a major food allergen. *J Allergy Clin Immunol* **104**, 1311–1320.
48. Sanchez-Monge, R., Pascual, C.Y., Diaz-Perales, A., Fernandez-Crespo, J., Martin-Esteban, M. & Salcedo, G. (2000) Isolation and characterisation of relevant allergens from boiled lentils. *J Allergy Clin Immunol* **106**, 955–961.
49. Beyer, K., Bardina, L., Grishina, G. & Sampson, H.A. (2002) Identification of sesame seed allergens by 2-dimensional proteomics and Edman sequencing: seed storage proteins as common food allergens. *J Allergy Clin Immunol* **110**, 154–159.
50. Wang, F., Robotham, J.M., Teuber, S.S., Tawde, P., Sathe, S.K. & Roux K.H. (2002) Ana o 1, a cashew (*Anacardium occidental*) allergen of the vicilin seed storage protein family. *J Allergy Clin Immunol* **110**, 160–166.
51. Teuber, S.S. & Petersen, W.R. (1999) Systemic allergic reaction to coconut (*Cocos nucifera*) in 2 subjects with hypersensitivity to tree nut and demonstration of cross-reactivity to legumin-like seed storage proteins: new coconut and walnut food allergens. *J Allergy Clin Immunol* **103**, 1180–1185.
52. Pasini, G., Simonato, B., Giannatasio, M., Gemgnani, C. & Curioni, A. (2000) IgE binding to almond proteins in two CAP-FEIA-negative patients with allergic symptoms to almond as compared to three CAP-FEIA-false-positive subjects. *Allergy* **55**, 955–958.
53. Yamauchi, F., Yamagishi, T. & Iwabuchi, S. (1991) Molecular understanding of heat-induced phenomena of soybean protein. *Food Rev Internat* **7**, 283–322.
54. Huang, L., Mills, E.N.C. & Morgan, M.R.A. (1998) Analysis of thermal stability of soy globulins using monoclonal antibodies. *Biochim Biophys Acta* **1388**, 215–226.
55. Mills, E.N.C, Huang, L., Noel, T.R., Gunning, A.P. & Morris, V.J. (2001) Formation of thermally-induced aggregates of the soy globulin β-conglycinin. *Biochim Biophys Acta* **1547**, 339–350.
56. Koppelman, S.J., Bruijnzeel-Koomen, C.A., Hessing, M. & de Jongh, H.H. (1999) Heat-induced conformational changes of Ara h 1, a major peanut allergen, do not affect its allergenic properties. *J Biol Chem* **274**, 4770–4777.
57. Gekko, K. & Timasheff, S.N. (1981) Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochem* **20**, 4677–4686.
58. Maleki, S.J., Chung, S.Y., Champagne, E.T. & Raufman, J.P. (2000) The effects of roasting on the allergenic properties of peanut proteins. *J Allergy Clin Immunol* **106**, 763–768.
59. Beyer, K., Morrow, E., Li, X.M. *et al*. (2001) Effects of cooking methods on peanut allergenicity. *J Allergy Clin Immunol* **107**, 1077–1081.
60. Santoro, L.G., Grant, G. & Pusztai, A. (1988) Differences in the degradation *in vivo* and *in vitro* of phaseolin, the major storage protein of *Phaseolus vulgaris* seeds. *Biochem Soc Trans* **16**, 612–613.
61. Tukur, H.M., Lalles, J.P., Mathis, C., Caugant, I. & Toullec, R. (1993) Digestion of soybean globulins, glycinin, β-Conglycinin and β-Conglycinin in the preruminant and ruminant calf. *Can J Anim Sci* **73**, 891–905.
62. Leibholz, J. (1981) Digestion in the pig between 7 and 35d of age 6. The digestion of hydrolysed milk and soya bean proteins. *Br J Nutr* **46**, 59–69.
63. Tukur, H.M., Pardal, P.B., Formal, M., Toullec, R., Lalles, J-P. & Guilloteau, P. (1995) Digestibility, blood levels of nutrients and skin responses of calves fed soyabean and lupin proteins. *Reprod Nutr Dev* **35**, 27–44.
64. Nielsen, S.S., Deshpande, S.S., Hermodson, M.A. & Scott, P.M. (1988) Comparative digestibility of legume storage proteins. *J Agric Food Chem* **36**, 896–902.
65. Kamata, Y. & Shibasaki, K. (1978) Formation of digestion intermediate of glycinin. *Agric Biol Chem* **42**, 2103–2109.
66. Plumb, G.W., Carr, H.J., Newby, V.K. & Lambert, N. (1989) A study of the trypsinolysis of pea 11S globulin. *Biochim Biophys Acta* **999**, 281–288.
67. Perez, D.M., Mills, E.N.C., Lambert, N., Johnson, I.T. & Morgan, M.R.A. (2000) The use of anti-soya globulin antisera in investigating soya digestion *in vivo*. *J Sci Food Agric* **80**, 513–521.
68. Kamata, Y., Otsuka, S., Sato, M. & Shubaki, K. (1982) Limited proteolysis of soyabean beta-conglycinin. *Agric Biol Chem* **46**, 2829–2834.
69. Gee, J.M., Wal, J.M., Miller, K. *et al*. (1997) Effect of saponin on the transmucosal passage of beta-lactoglobulin across the proximal small intestine of normal and beta-lactoglobulin-sensitised rats. *Toxicol* **117**, 219–228.

70. Shimoyamada, M., Ikedo, S., Ootsubo, R. & Watanabe, K. (1998) Effects of soybean saponins on chymotryptic hydrolyses of soybean proteins. *J Agric Food Chem* **46**, 4793–4797.
71. Kroll, J., Rawel, H.M., Rohn, S. & Czajka, D. (2001) Interactions of glycinin with plant phenols – influence on chemical properties and proteolytic degradation of the proteins. *Nahrung/Food* **45**, 388–389.
72. Bourdillon, J. (1951) A crystalline bean seed protein in combination with phytic acid. *J Biol Chem* **189**, 65–72.
73. Stanley, J.S. & Bannon, G.A. (1999) Biochemistry of food allergens. *Clin Rev Aller Immunol* **17**, 279–291.
74. Maleki, S.J., Kopper, R.A., Shin, D.S. *et al*. (2000) Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J Immunol* **164**, 5844–5849.
75. Diep, O., Boulet, M. & Castaigne, F. (1982) Effects of extreme pH and salt concentration on intrinsic viscosity and Huggins' constant (k') of 7S and 11S soybean globulins. *Can Inst Food Sci Technol J* **15**, 316–318.
76. Beardslee, T.A., Zeece, M.G., Sarath, G. & Markwell, J.P. (2000) Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *Int Arch Allergy Immunol* **123**, 299–307.
77. Paaren, H.E., Slightom, J.L., Hall, T.C., Inglis, A.S. & Blagrove, R.J. (1987) Purification of a seed glycoprotein: *N*-terminal and deglycosylation analysis of phaseolin. *Phytochem* **26**, 335–343.
78. Yamada, T., Aibara, S. & Morita, Y. (1981) Reconstitution of arachin I from isolated subunits. *Agric Biol Chem* **45**, 1243–1250.
79. Kumar, K.G. & Venkatamaran, L.V. (1980) Chickpea seed proteins: isolation and characterisation of 10.3 S protein. *J Agric Food Chem* **28**, 524–529.
80. Patil, S.P., Niphadkar, P.V. & Bapat, M.M. (2001) Chickpea: a major allergen in the Indian subcontinent and its clinical and immunochemical correlation. *Ann Allergy Asthma Immunol* **87**, 140–145.
81. Melo, T.S., Fereira, R.B. & Teixeira, A.N. (1994) The seed storage proteins from *Lupinus albus*. *Phytochem* **37**, 641–648.
82. Carr, H.J., Plumb, G.W., Parker, M.L. & Lambert, N. (1990) Characterisation and crystallisation of an 11S seed storage globulin from coconut. *Food Chem* **38**, 11–20.
83. Roux, K.H., Teuber, S.S., Robotham, J.M. & Sathe, S.K. (2001) Detection and stability of the major almond protein allergen in foods. *J Agric Food Chem* **49**, 2131–2136.
84. Kamiya, N., Sakabe, K., Sakabe, N., Sasaki, K., Sakakibara, M. & Noguchi, H. (1983) Structural properties of Brazil nut 11S globulin, Excelsin. *Agric Biol Chem* **47**, 2091–2098.
85. Pastorello, E.A., Farioli, L., Pravettoni, V. *et al*. (1998) Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. *J Allergy Clin Immunol* **102**, 1021–1027.
86. Yuno, N., Matoba, T., Hirose, M. & Hasegawa, K. (1986) Subunit structure of sesame 13S globulin. *Agric Biol Chem* **50**, 983–988.
87. Tai, S.S.K., Lee, T.T.T., Tsai, C.C.Y., Yiu, T.J. & Tzen, J.T.C. (2001) Expression and deposition of three storage proteins, 11S globulin, 2S albumin and 7S globulin in maturing sesame seeds. *Plant Physiol Biochem* **39**, 981–992.
88. Hasegawa, K., Murata, M. & Fujino, S. (1978) Characterisation of subunits and temperature-dependent dissociation of 13S globulin of sesame seed. *Agric Biol Chem* **42**, 2291–2297.
89. Blagrove, R.J. & Lilley, G.G. (1980) Characterisation of cucurbitin from various species of the cucurbitaceae. *Eur J Biochem* **103**, 577–584.
90. Figueredo, E., Cuesta-Herranz, J., Minguez, A. *et al*. (2000) Allergy to pumpkin and cross-reactivity to other *Cucurbitaceae* fruits. *J Allergy Clin Immunol* **106**, 402–403.
91. Sathe, S.K., SzeTao, K.W.C., Wolf, W.J. & Hamaker, B.R. (1997) Biochemical characterization and *in vitro* digestibility of the major globulin in cashew nut (*Anacardium occidentale*). *J Agric Food Chem* **45**, 2854–2860.

# 10 The Role of Common Properties in Determining Plant Food Protein Allergenicity

E.N. CLARE MILLS, JOHN A. JENKINS and PETER R. SHEWRY

## 10.1 Introduction

The molecular and cellular mechanisms involved in triggering the production of IgE responses characteristic of type I allergy are not completely understood at present. However, it is clear that the pathogenesis of allergic disease has two phases. The first involves sensitisation of a naïve immune system to produce an IgE response towards an allergen, whilst in the second phase further exposure to the same allergen results in elicitation of an allergic reaction, together with further sensitisation and modulation of the immune response. It is also apparent that a number of factors, such as the level of exposure and the properties of an allergen, together with the genetic predisposition of individuals to atopy, all play an important role in these phases. As a consequence of the sequencing of the human genome, we are now beginning to identify some of the genetic factors involved in allergic disease [1]. Our increased understanding of the structural attributes and biological activities of allergens, together with route of exposure, also enables us to begin addressing the issues regarding allergy-inducing agents, the allergens.

The preceding chapters of this book have given an overview of the structural characteristics and biological properties of the major plant food protein allergen families that have been identified to date. From our current state of knowledge it is evident that some allergens, such as the Bet v 1 family, generally sensitise by inhalation. Partly as a consequence of their abundance in pollen, Bet v 1 family members are major pollen allergens, which can go on to trigger food allergies because conserved homologues are also present in widely consumed fruits and vegetables. In contrast other allergen families, such as the seed storage globulins, appear to sensitise individuals primarily through the gastrointestinal (GI) tract, perhaps because they are generally found in foods and not inhaled particulates. Other allergens such as the nsLTPs, the α-amylase/trypsin inhibitor family and the cysteine proteases can sensitise individuals both by inhalation and orally, via the gut. This is because many of these proteins are present both in food and in some cases pollen (e.g. nsLTPs), and others are found in inhaled dusts (e.g. α-amylase/trypsin inhibitor family). Finally, latex allergens may initially sensitise via contact with tissues (e.g. during surgery and catheterisation) and then lead to dietary allergies to related proteins in fruits and vegetables.

The ability to sensitise by different routes is also a characteristic of allergens of animal origin, such as the lipocalin superfamily allergens. Lipocalin proteins from animal danders and urine sensitise via the lungs, whilst the cows' milk allergen β-lactoglobulin sensitises via the GI tract [2]. Thus, the route of exposure is clearly an important factor in determining protein allergenicity in general. But is this the only

factor of importance? Are there structural attributes and biological properties shared by allergens and can these give us clues as to whether certain proteins are more likely to trigger an allergic reaction than others?

A summary of the common properties of the different plant food allergen families is given in Table 10.1. The following discussion considers how the structures and properties of plant food allergen families may relate to certain plant proteins becoming food allergens, either by maximising exposure of the immune system to immunologically active proteins and/or fragments or by being inherently more effective at triggering an IgE response in susceptible individuals.

## 10.2 Allergen exposure via the gastrointestinal tract

For a food allergen to sensitise via the GI tract it must possess certain structural and biological attributes which preserve its structure from the destructive effects of that organ, including resistance to low pH, proteolysis and surfactants such as bile salts. Whilst there are some exceptions, the vast majority of those allergens thought to sensitise via the GI tract belong to either the prolamin superfamily (comprising the prolamin storage proteins of cereals, nsLTPs, 2S albumins and the α-amylase inhibitors) or the cupin superfamily (comprising the 11S legumin-like and 7S vicilin-like seed storage globulins). All these allergens generally share two properties which enable them to survive digestion: abundance and structural stability. This combination of properties may help ensure that enough of the protein survives in a sufficiently intact form to be taken up by the gut and sensitise the mucosal immune system. However, whilst abundance is an important factor, it is probably secondary to protein stability. Thus ribulose-1, 5-bisphosphate carboxylase/oxygenase (usually abbreviated to rubisco), accounts for about 30–40% of total leaf protein in most species, but has never been found to be an allergen, while nsLTPs have been designated as pan-allergens [3] and yet are generally minor components in edible plant tissues [4]. The properties of abundance and stability are also shared by food allergens of animal origin which sensitise by ingestion, and include milk allergens such as cows' milk β-lactoglobulin [5], the fish allergen parvalbumin [6] and hen egg ovomucoid [7]. These allergens are also abundant, thermostable and resistant to proteolysis. So how might the biological activities and the three-dimensional structures of allergens confer stability to unfolding and resistance to proteolysis?

### 10.2.1 *Protein stability to unfolding*

Several factors are thought to contribute to protein stability including a compact three-dimensional structure, ligand binding and glycosylation. These are relevant to both the resistance of proteins to denaturation by food processing, such as cooking, and stability to extremes of pH such as those experienced in the GI tract. It is evident that there is no single stability-determining factor for proteins, as nature has used a breadth of strategies to develop stable proteins, including adaptive mutations [8]. Some of the major factors involved in determining protein stability are described below.

**Table 10.1** Common properties of the different plant food allergen families

| Plant allergen family | Common properties |
|---|---|
| **Prolamin superfamily** | · Mostly small polypeptides with $M_r$ less than 20 000 which adopt a characteristic fold consisting of bundles of four α-helices stabilised by disulphide bonds.<br>· α-Helical proteins which contain a high proportion of cysteine residues and are highly disulphide bonded. There are almost always eight cysteines in the pattern C . . . C . . . CC . . . CXC . . . C . . . C and often two additional cysteines making a fifth disulphide. The CC pair make disulphide bonds conserved across the superfamily, whilst those formed by the disulphides from CXC cysteines have various different topologies.<br>· Resistant to proteolysis.<br>· Stable to low pH.<br>· Despite the compact disulphide bonded core, there are often a number of flexible loops, which may be important antibody recognition sites.<br>· Many are able to bind lipid in a pocket, which can increase resistance to proteolysis or denaturation.<br>· Many members are able to bind lipid droplets, adsorbing to oil:water interfaces (i.e. act as an emulsifier).<br>· Many are abundant in the plant tissue that is eaten.<br>· Many have a role in plant defence against pathogens. |
| **Cupin superfamily** | · Large oligomeric β-barrel proteins of $M_r$ 120–360 000.<br>· Thermostable and able to form thermally induced large aggregates.<br>· Resistant to proteolysis, forming large stable intermediates.<br>· Stable to low pH.<br>· Able to bind lipid droplets (i.e. act as an emulsifier) especially following thermal denaturation.<br>· Abundant in edible seeds. |
| **Cysteine protease family** | · Medium sized ($M_r$ 25–30 000) two domain proteins which are made as larger $M_r$ 35–45 000 precursors.<br>· Stabilised by three disulphide bonds and resistant to proteases.<br>· Most are proteases (except for the soybean allergen Gly m BD30k), an activity which may contribute to allergenicity, as has been shown for the dust mite allergen, Der p 1.<br>· Several have a function in plant defence against pathogens. |
| **The Bet v 1 family of cross-reactive pollen-fruit/ vegetable allergens** | · $M_r$~17 000 proteins which are readily soluble in water or isotonic salt solutions.<br>· Readily broken down by proteases.<br>· Generally thermolabile and frequently possess conformational IgE epitopes which are destroyed by heating.<br>· Plant defence proteins of the PR10 family.<br>· Binds lipids, generally steroids. |
| **The profilin family of cross-reactive pollen-fruit/ vegetable allergens** | · Approximately $M_r$~14 000 proteins which are readily soluble in water or isotonic salt solutions.<br>· Readily broken down by proteases.<br>· Moderately thermostable, frequently possess conformational IgE epitopes which are destroyed by heating.<br>· Function in the plant as actin-binding proteins.<br>· Less conserved than the Bet v 1 family across plant species and thus shows lower IgE cross-reactivity between pollen profilin and other homologues found in fruits and vegetables. |

| | |
|---|---|
| **Cross-reactive latex-fruit allergens with hevein-like chitin-binding domains** | · Presence of a chitin-binding domain homologous with that of latex hevein or Hev b 6. Hevein is a PR4 protein because of its chitinase-like *C*-terminal domain. However, the cross-reaction is to the *N*-terminal chitin-binding domain, Hev b 6.02.<br>· The chitin-binding domain is short and disulphide rich (eight conserved cysteines in 42 residues).<br>· The chitin-binding domain appears sufficiently stable to survive vulcanisation at 100–120°C but not production of dry rubber (such as tyres) at 140–160°C.<br>· Well conserved across species with >70% identity between rubber and several plant food species. |

10.2.1.1 *Three-dimensional scaffold.* The three-dimensional architecture of a protein is determined by the way in which the polypeptide chain is folded which is in turn driven by thermodynamic and kinetic considerations. Thermodynamic aspects of protein folding are encapsulated by the historic studies of Anfinsen [9] which demonstrated that the three-dimensional structure of a protein is encoded in some way by its amino acid sequence. This observation is best explained by native folded structures adopting a global free energy minimum, the depth of this well determining the thermodynamic stability of a protein. For a range of globular proteins, the free energy difference between the native and denatured state can be as low as 14 kJ mole$^{-1}$, with metabolically important proteins such as protein G and ubiquitin having $\Delta G$ values of around 24–35 kJ mole$^{-1}$ [10]. For more stable proteins, such as camelid (i.e. camel and llama) single chain antibody fragments $\Delta G$ is in the order of 30–60 kJ mole$^{-1}$ [11]. No single structural motif is associated with stability, although proteins from thermophilic organisms (which are able to grow at elevated temperatures) have a greater propensity to adopt β-structures, implying they are possibly more thermostable structural elements than α-helices, although in thermophiles the latter are found to have stronger charge dipoles, which can stabilise such structures [12].

10.2.1.2 *Disulphide bonds.* Protein engineering approaches to improving protein thermostability indicate that while stability is generally associated with increased numbers of disulphide bridges, the relationship is complex. Thus, disulphide bonds can contribute to the stability of both the native and denatured form of a protein, the overall protein stability arising from a balance between the stabilities of these different states [13]. In general, both intra-and inter-chain disulphide bonds constrain the three-dimensional scaffold such that perturbation of this structure by heat or chemical means is limited and frequently reversible.

10.2.1.3 *Size.* Comparisons of proteins in thermophilic and mesophilic organisms have shown that soluble proteins are on average smaller in thermophiles. It has been suggested that smaller proteins would be more thermostable by virtue of their lower heat capacity [12] and/or that the shortened loops in the smaller proteins lead to a smaller difference in entropy between the folded and unfolded states, thus stabilising the former [14].

10.2.1.4 *Glycosylation and glycation.* It is now evident that *N*-glycosylation can have a significant stabilising effect on protein structure [15]. Thus, glycosylation of

the 7S globulin of peas increases trimer stability and resistance to chemical denaturation [16]. Food processing also results in covalent modification of proteins, with the formation of Maillard adducts amongst many types of modifications which may either increase or reduce protein stability. In the Maillard reaction, sugars modify the amino groups on proteins to form Amadori compounds which may then rearrange to produce a range of adducts, known as advanced glycation/glycosylation end products (AGE). As well as forming during thermal processing, and particularly during the application of dry heating procedures such as roasting, AGE are slowly formed over days and months as a consequence of the ageing process in both foods and biological systems. Such products have been shown to destabilise the quaternary structure of proteins, such as reducing the stability at low pH of the triple helix of collagen [17].

10.2.1.5 *Interactions with lipids.* Proteins such as the lipocalins and nsLTPs which possess a lipid-binding pocket show increased stability when the pocket is occupied. Thus, the thermostabilty of β-lactoglobulin increases on lipid binding [18] as does that of the nsLTP of wheat [19].

Many plant food allergens are also able to associate with lipid structures and membranes. For example, proteins belonging to the prolamin and cupin superfamilies can bind to membranes and/or act as emulsifiers by adsorbing to lipid droplet surfaces. Such activity has been demonstrated for nsLTPs [20], 2S albumins [21, 22] and the 11S and 7S globulins from legumes such as soy [23]. Such associations may either occur naturally in the food, because of processing (which generally potentiates the emulsifying properties of proteins) or in the GI tract because of the digestive process. There is also evidence that proteins associated with lipid bilayers have enhanced thermal stability [24] and it may be that such lipid-associated proteins may be protected from the degradative environment in the gut. It is also possible that lipid binding may assist allergens in being taken up by the gut mucosa, either through association with cell membranes as has been suggested for 2S albumins, or by co-adsorption on the surface of lipid droplets [21].

### 10.2.2 *Protein stability and resistance to proteolysis*

A number of allergenic proteins, including those from peanut, soybean and cows' milk, show remarkable resistance to digestion by pepsin when compared with non-allergenic proteins. In one study all of the allergenic proteins tested remained either undigested, or gave stable fragments, which persisted for 8–60 minutes (depending upon the allergen), while the non-allergens were completely digested after less than 15 seconds [25]. As peptides require a molecular weight of greater than 3000 daltons in order to stimulate an immune response, large stable fragments, as well as intact proteins, have the potential to act as sensitisers. One way of viewing the role protein structure plays in potentiating allergenicity is to consider those factors that make them poor substrates for proteases, although it may be important to distinguish between *nicking* and degradation [26]. Some proteins are able to retain much of their tertiary and quaternary structure even after digestion by proteases, the seed storage globulins being a good example of this [27]. Several factors are thought to be involved in susceptibility to proteolysis and are outlined below. Figure 10.1 contrasts the easily digested Mal d 1 with Zea m 14, which is stable to proteolysis probably because its four disulphides limit the effect of nicking to very local unfolding without creating new sites.

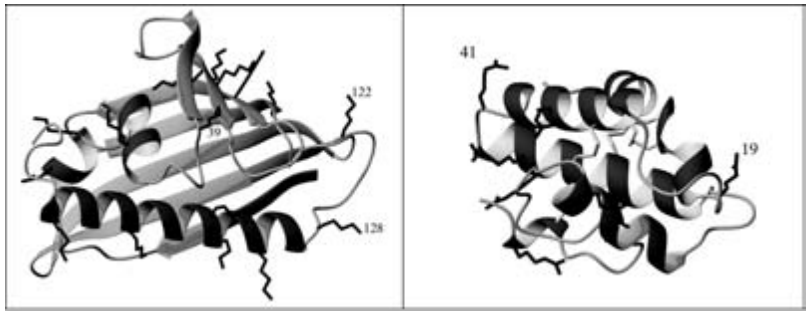


**Fig. 10.1** A comparison of potential tryptic cleavage sites (i.e. arginine and lysine residues) for Mal d 1, the Bet v 1 homologue of apple susceptible to proteolysis, and Zea m 14, an nsLTP allergen from maize which is stable to proteolysis. Arginine and lysine residues are shown as dark stick drawings with ribbon representations of two allergens. On the left a model of the main apple allergen Mal d 1b, built from the coordinates of the closely similar Bet v 1L (1FM4, 65), is shown with the lysines 39, 122 and 128, which are not involved in secondary structure, labelled. On the right the maize allergen Zea m 14 (1MZL, 66), is shown with arginines 19 and 41, which are not involved in secondary structure, labelled.

10.2.2.1 *Stability.* Comparisons of proteolytic susceptibility of proteins from thermophiles and mesophiles, as well as engineered proteins, have shown that resistance to proteolysis and stability to temperature and denaturants are correlated. However, this correlation between stability and proteolysis is imperfect, as whilst it is also possible some engineered proteins have been produced which are stable to denaturation but not resistant to proteolysis [28, 29]. Such proteins are probably resistant to global unfolding, but can still undergo local unfolding around the proteolytic cleavage sites. The importance of local unfolding is supported by protein engineering studies which have succeeded in producing extremely stable enzymes by avoiding it [30]. Whilst the relationship between protein stability and rigidity is still a matter of debate [31–33], it is clear that stable proteins have a low unfolding rate [34]. In the industrially important case of proteases, the situation is simplified and thermal stability correlates perfectly with stability against autolysis, causing proteases to be significantly different from other proteins in being more compact and rigid [35]. Thus, it is clear that stable proteins, as well as frequently being more resistant to proteolysis *per se*, will also resist denaturation, either following food processing or in the GI tract environment. Consequently, they will make poor substrates for proteases compared with those that unfold and hence have more mobile polypeptide backbones. Factors that are known to further enhance protein stability, such as ligand binding will also render them even more resistant to digestion by proteases.

10.2.2.2 *Flexibilty.* During limited proteolysis of proteins only a few of the possible proteolytic cleavage sites (i.e. the most susceptible) are actually cut. A structural analysis of such sites for the enzymes trypsin [36] and thermolysin [37] has revealed that they tend to be located in regions with greater atomic mobility, as indicated by the higher temperature factors determined by X-ray crystallography. Proteolysis is also favoured in exposed loops rather than in β-sheets or α-helices, and cleavage sites can be predicted with fair confidence from structures [38]. Aspartyl proteases, such as pepsin, require a certain degree of flexibility in their substrates to allow a stretch of polypeptide chain 6–8 residues in length to lie across the active site in an extended conformation.

10.2.2.3 *Sequence specificity.* Endopeptidases usually have some specificity regarding the sequences they will cleave. Pepsin preferentially attacks between pairs of large hydrophobic residues but has significant secondary specificity for more distant residues. Trypsin and its homologues show strong specificity for residues before the peptide bond to be cleaved. Thus, trypsin cleaves at the *C*-terminal side of the basic residues, arginine and lysine, whilst the less specific proteases, chymotrypsin and elastase, cleave at the *C*-terminal side of either phenylalanine (chymotrypsin) or small aliphatic residues (elastase) [39]. Further down the digestive tract, food proteins also encounter a battery of exopeptidases, including both amino- and carboxy-peptidases which merely require access to a free *N*- or *C*-terminal residue in a substrate protein. Consequently, susceptibility to proteolysis by exopeptidases is limited by the number and accessibility of the *N*- and *C*-termini. As food proteins are nicked by endopeptidases in the GI tract, more *N*- and *C*-termini are liberated increasing the rates of proteolysis due to exopeptidases.

10.2.2.4 *Steric hindrance.* In order for a protein to be broken down by a protease, the latter must be able to approach and bind to the putative cleavage site. Steric factors can block this event, and hence reduce rates of proteolysis. For example, glycosylation is thought to protect proteins from proteolysis through shielding of potential cleavage sites by the carbohydrate moieties. However, there is also evidence that glycosylation increases the stability of the native conformation, and this, rather than steric considerations, may be responsible for the resistance to proteolysis shown by some glycoproteins [40]. In addition to glycosylation, processing-induced glycation may either protect or promote protein digestion [17, 41]. As glycation involves modification of basic lysine and arginine residues, proteolysis by trypsin may be reduced simply through blocking of protease cleavage sites [41]. However, the effects on proteolysis by enzymes with other specificities are complex and may be related to both modification of the protein three-dimensional structure and the cross-linking effects of glycation. Unfolding may be necessary for a protease to gain access to putative cleavage sites, especially for those enzymes specific for more hydrophobic residues. Such residues are generally buried within the interior of a protein molecule, or for amino- and carboxy-peptidases which require access to either the *N*- or *C*-termini of a protein, or the new termini introduced by nicking of the polypeptide chain by endopeptidases. Thus, denaturation of food proteins by cooking procedures together with glycation and Maillard modification may potentiate proteolysis. However, this may be offset by the steric hindrance effects which result from the formation of thermally induced protein aggregates, coupled with glycation and Maillard-induced cross-linking of proteins.

## 10.3 Allergen exposure via the lungs

The factors involved in sensitisation and elicitation of allergic reactions via the lungs are much more straightforward than is the case for exposure via the GI tract, as the agents that come into contact with the immune system are not modified by food processing and digestion. Where food allergies result from prior sensitisation via the lungs, as is thought to be the case for allergies involving Bet v 1 homologues, profilins, and class I chitinases, the cross-reactive food allergens have similar characteristics to those of inhalant allergens. These include a ready solubility in dilute salt solutions

which allow allergen solubilisation from inhaled particulates in the liquid layers lining the lungs, which may be accompanied by proteolytic activity which assists allergens to enter the body by permeabilising the lung lining [42].

Not all inhalant allergens cause cross-reactive allergy syndromes. Those that do, share a high degree of conserved surface residues (some times even whole domains) with homologous proteins present in edible vegetative tissues of unrelated plant species. Thus, the apple allergen Mal d 1, which is involved in the birch pollen-fruit allergy syndrome, does not stimulate the formation of anti-Mal d 1 IgE following ingestion of apple fruit. Instead, because of its high degree of homology with Bet v 1, Mal d 1 can bind to anti-Bet v 1 IgE associated with mast cells, triggering histamine release and eliciting an allergic reaction. Like many inhalant allergens, Mal d 1 is thermolabile and readily digested by proteases. Whilst it is intrinsically able to stimulate an IgE response, its lack of stability in food and the GI tract means that Mal d 1 is unable to sensitise an individual through ingestion. The ability only to elicit an allergic reaction but not sensitise an individual, has led such food allergens to be considered *incomplete* allergens [43]. Their lack of stability also means that reactions cross-reactive allergens in fruits and vegetables tend to be confined to the oral cavity. Similarly, it has been suggested that individuals, who suffer from the occupational cereal allergy known as Bakers' asthma, can tolerate cereal-containing foods, because the α-amylase and trypsin inhibitors, which are the major inhalant allergens, are modified by baking [44].

## 10.4 Intrinsic allergenicity and plant food allergens

Implicit in their ability to stimulate IgE synthesis, is the fact that all allergens must *per se* be able to act as good antigens, eliciting a high affinity-sustained antibody response. Thus, it is well known that proteins and peptides need to have a molecular weight of around 4–6000 daltons in order to ellicit any form of antibody response, irrespective of isotype [45]. The area of a molecule recognised by the binding site of an antibody (also known as a paratope) is termed an epitope. The steric fit between an epitope and a paratope can vary, the goodness of fit determining antibody affinity ($K_a$), which can range between $10^6$ and $10^{11}$ L/mol. In general, the affinity of IgE antibodies is also high and recent studies using human allergic sera from ragweed and dust mite-allergic individuals have shown them to contain between two and three populations of different IgE affinities of $10^8$–$10^{11}$ L/mol [46]. In dust mite these different populations of IgE recognise different epitopes [47] indicating that for this allergen at least, that affinity maturation of the IgE response is heterogeneous, with one IgE epitope being immunodominant.

Epitopes have been classified as being either continuous (also termed sequential or linear) or discontinuous (also termed conformational) in nature. For the former the antibody-binding site comprises a linear stretch of amino acid sequence of 6–14 residues in length, whilst conformational epitopes comprise regions of the linear sequence of a protein which are brought together spatially as a consequence of the way a protein is folded [45]. It is thought that the majority of protein epitopes are conformational in nature, although continuous epitopes are found in both native and denatured proteins. Detailed epitope analysis of the pollen allergen Bet v 1 indicates that the major IgE epitopes are conformational in nature [48] covering a large area of the surface of Bet v 1 and its

homologues in fruits and vegetables. It is evident that conformational epitopes are also important in a wide number of both inhalant and food allergens [48].

Overlapping synthetic peptides have been used extensively in epitope mapping studies on allergens that are thought to sensitise via the GI tract, including those from peanut, soy and walnut [49–53]. Whilst some data are confusing to interpret (e.g. some studies having shown IgE binding to pro-sequences which are removed post-translationally and are not found in the mature protein) and only continuous epitopes can be identified using this methodology, it is clear that IgE epitopes are distributed throughout the protein, including internal protein structures. These data suggest that sensitisation to these allergens involves denatured rather than native protein species, as might be anticipated since they are generally consumed in processed foods. This is in contrast to the cross-reactive allergens, where sensitisation occurs to the native protein, and thermal treatment and aggregation generally result in the destruction of IgE epitopes.

The presence of multiple epitopes on an allergen, coupled with the ability to retain them following food processing and digestion, is crucial to the ability of food allergens to trigger cross-linking of membrane-bound IgE on mast cells. This in turn results in the release of inflammatory mediators (such as histamine), which cause the symptoms manifested in an allergic episode. Thus, an allergen must be at least 20–25 amino acid residues in length in order to accommodate a minimum of two antibody-binding sites and hence be *functional* in cross-linking mast cell IgE. The importance of epitope density and orientation is illustrated by the fact that trimeric forms of the pollen allergen Bet v 1 are more effective at releasing histamine than the monomeric form [54]. Factors that alter the distribution of IgE epitopes on an allergen when it finally encounters the immune system, such as processing-induced aggregation, will clearly affect allergenic potency although this has yet to be demonstrated for any food allergens.

Chemical substituents on proteins such as the *N*-linked oligosaccharides, $Man_{5–6}GlcNAc_2$ and $Man_{3–4}XylGlcNAc_2$, present on the major peanut allergen Ara h 1 also have the potential to act as thermostable epitopes [55]. However, because of their sparsity on the surface of a protein, such epitopes may not be able to cross-link IgE and hence trigger histamine release. Consequently, the role such carbohydrate epitopes may play in allergic disease is unclear [56].

Despite much study, we have little information regarding the role that allergen structure might play in triggering the production of specific IgE, rather than IgG. There is little evidence that particular protein sequences or structures promote the development of an IgE response although food allergens appear to be good antigens (i.e. able to stimulate an antibody response). However, some evidence from animal studies, based on a limited number of allergens tested so far, indicates that allergens are intrinsically able to preferentially elicit an IgE response [57, 58]. It may be that allergens are also preferentially taken up by the immune systems, through the ability of certain allergens to interact with cell membranes, or through the presence of AGE, which may activate the immune system through AGE receptors.

## 10.5 Flexible and mobile protein structures

Much of our view on protein structure and its role in determining the function and properties of a protein, has been based on the proliferation of 3D structures now

available from X-ray crystallography and high resolution NMR. This is reflected in the information and discussion presented in this book, which has focused around plant food allergens for which well-defined structures have been obtained. But what about proteins with highly mobile structures which are not amenable to such structural studies? It is becoming increasingly evident that flexibility is an important part of the correct functioning of some proteins [59]. Are any of them allergens? What does structural stability mean for a protein, which is inherently flexible? Such flexible proteins should be inherently good substrates for endoproteases, making them readily broken down during digestion, posing a challenge to the premise that stability is an important factor in predisposing a protein to becoming an allergen.

Of the food allergens that have been identified to date, only the seed storage prolamins of cereals and the caseins of milk appear to fall into this classification of flexible proteins. The central repetitive domain of seed storage prolamins is highly mobile existing as an ensemble of conformations in a dynamic equilibrium of what approximates to a mixture of poly-proline II and β-turn type structures. These structures have been found to be highly immunogenic [60] possibly because β-turn structures make very effective epitopes [61]. This coupled with the high proportion of proline and the renowned insolubility of these proteins in physiological solutions may account for their ability to act as allergens. However, the reasons underlying the ability of casein, one of the most digestible food proteins known [62], are more difficult to account for. Many studies investigating the breakdown and allergenic activity of casein have not addressed the fact that in many dairy products consumed by man, caseins are presented in a complex micellar form, which aggregates into extensive networks in foods such as yoghurts. There is also evidence that complexation with calcium forms more stable domains, although their structure is still a matter of debate [63]. Only when we have a more complete understanding of the role that food structure, rather than purified individual proteins, has on the breakdown of allergenic proteins and their subsequent presentation to the immune system we will be able to begin to arrive at a more complete explanation as to how the structural properties of proteins may influence their allergenic properties.

## 10.6 Conclusions

At present it appears that the structural features and properties of globular proteins probably do play a role in predisposing them to becoming allergens, at least because such properties ultimately determine the exposure of the immune system to immunologically active protein or derived fragments. These features relate to the abundance of an allergen in a food, its overall thermal and proteolytic stability and the ability to effectively elicit an antibody response. It has previously been noted that a remarkably large number of plant food allergens are also involved in plant protection, including the so-called pathogenesis-related (PR) proteins [64]. It is possible that PR proteins are particularly allergenic as they also require high stability to survive digestion by proteases secreted by the fungi at the site of infection.

Analysis of the common properties of plant food allergens indicates that, in the same way that only a limited number of foods are responsible for the majority of food

allergies, the allergens responsible also belong to only a limited number of protein families. These protein families clearly have common properties, of which their high degree of stability is particularly notable. This may potentiate their ability to act as allergens, both with regard to sensitisation and elicitation of allergic reactions. However, it is clear that these properties are not the sole determinant of allergenic potential, and it remains to be determined why one type of protein is allergenic in some foods but not others. For example, in Brazil nut, the major allergen is a 2S albumin, the 11S globulin not having been reported as an allergen, while both 7S and 11S globulins are allergens in peanut and soybean. In other widely consumed legumes such as peas and beans neither class of seed storage proteins has been identified as an allergen. It is possible that additional factors present in some foods promote allergenicity, perhaps by exerting an adjuvant-like effect on the immune system. The identification and characterisation of such factors is crucial for our ability to predict the allergenicity of foods. In the meantime, our increasing understanding of the role that protein structure and properties play in predisposing certain types of proteins to becoming allergens brings this possibility much closer.

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## References

1. Heinzman, A. & Deichman, K.A. (2001) Genes for atopy and asthma. *Curr Opin Allergy Clin Immunol* **1**, 387–392.
2. Mantyjarvi, R., Rautiainen, J. & Virtanen, T. (2000) Lipocalins as allergens. *Biochim Biophys Acta* **1482**, 308–317.
3. Diaz-Perales, A., Laombardero, M., Sanchez-Monge, R. *et al*. (2000) Lipid-transfer proteins as potential plant panallergens: cross-reactivity among proteins of *Artemisia* pollen, *Castanea* nut and Rosaceae fruits, with different IgE-binding capacities. *Clin Exp Allergy* **30**, 1403–1410.
4. Douliez, J.-P., Michon, T., Elmorjani, K. & Marion, D. (2000) Structure, biological and technological function of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J Cereal Sci* **32**, 1–20.
5. Wal, J.-M. (1998) Cow's milk allergens. *Allergy* **53**, 1013–1022.
6. Bugajska-Schretter, A., Grote, M., Vangelista, L. *et al*. (1999) Purification, biochemical, and immunological chracterisation of a major food allergen: different immunoglobulin E recognition of the apo- and calcium-bound forms of carp parvalbumin. *Gut* **46**, 661–669.
7. Cooke, S.K. & Sampson, H.A. (1997) Allergenic properties of ovomucoid in man. *J Immunol* **159**, 2026–2032.
8. Fields, P.A. (2001) Protein function at thermal extremes: balancing stability and flexibility. *Comp Biochem Physiol* Part A **129**, 417–431.
9. Anfinsen, C.B. (1973) Principles which govern the folding of protein chains. *Science* **181**, 223–230.
10. Makhatadze, G.I. & Privalov, P.L. (1995) Energetics of protein structure. *Adv Protein Chem* **47**, 307–425.
11. Dumoulin, M., Conrath, K., van Meirhaeghe, A. *et al*. (2002) Single-domain antibody fragments with high conformational stability. *Protein Sci* **11**, 500–515.
12. Chakravarty, S. & Varadarajan, R. (2000) Elucidation of determinants of protein stability through genome sequence analysis. *FEBS Lett* **470**, 65–69.
13. Zavodszky, M., Chen, C.W., Huang, J.K., Zolkiewski, M., Wen, L. & Krishnamoorthi, R. (2001) Disulfide bond effects on protein stability: designed variants of *Cucurbita maxima* trypsin inhibitor-V. *Protein Sci* **10**, 149–160.

14. Thompson, M.J. & Eisenberg, D. (1999) Transproteomic evidence of a loop-deletion mechanism for enhancing protein thermostability. *J Mol Biol* **290**, 595–604.
15. Wormald, M.R. & Dwek, R.A. (1999) Glyoproteins: glycan presentation and protein-fold stability. *Struct* **7**, R155–R160.
16. Pedrosa, C., De Felice, F.G., Trisciuzzi, C. & Ferreira, S.T. (2000) Selective neoglycosylation increases the structural stability of vicilin, the 7S storage protein of pea seeds. *Arch Biochem Biophys* **15**, 203–210.
17. Tian, S.-F., Toda, S., Higashino, H. & Matsumura, S. (1996) Glycation decreases the stability of the triple-helical strands of fibrous collagen against proteolytic degradation by pepsin in a specific temperature range. *J Biochem* **120**, 1153–1162.
18. Creamer, L.K. (1995) Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine β-lactoglobulin. *Biochemistry* **34**, 7170–7176.
19. Douliez, J.-P., Jegou, S., Pato, C., Mollle, D., Tran, V. & Marion, D. (2001) Binding of two monoacylated lipid monomers by the barley lipid transfer protein, LTP1, as viewed by fluorescence, isothermal itrationcalorimetry and molecular modelling. *Eur J Bioch* **268**, 384–388.
20. Subriade, M., Salesse, D., Marion, D. & Pézolet, M. (1999) Interaction of non-specific wheat lipid transfer protein with phospholipid monolyers imaged by fluorescence microscopy and studied by infrared spectroscopy. *Bipohys J* **69**, 974–988.
21. Onaderra, M., Monsalve, R.I., Mancheno, J.M. *et al*. (1994) Food mustard allergen interaction with phospholipid vesicles. *Eur J Biochem* **225**, 609–615.
22. Burnett, G.R., Wickham, M., Fillery-Travis, A. *et al*. (2002) The interactions between protein allergens and model gastric emulsions. *Biochem Soc Trans* **20**, 916–918.
23. Kinsella, J.E. (1979) Functional properties of soy proteins. *J Am Oil Chem Soc* **56**, 242–258.
24. Taneva, S.G., Donchev, A.A., Dimitrov, M.I. & Muga, A. (2000) Redox- and pH-dependent association of plastocyanin with lipid bilayers: effects on protein conformation and thermal stability. *Biochim Biophys Acta* **1463**, 429–438.
25. Astwood, J.D., Leach, J.N. & Fuchs, R.L. (1996) Stability of food allergens to digestion *in vitro*. *Nature Biotechnology* **14**, 1269–1273.
26. Bodi, A., Kaslik, G., Venekei, I. & Graf, L. (2001) Structural determinants of the half-life and cleavage site preference in the autolytic inactivation of chymotrypsin. *Eur J Biochem* **268**, 6238–6246.
27. Plumb, G.W., Carr, H.J., Newby, V.K. & Lambert, N. (1989) A study of the trypsinolysis of pea 11S globulin. *Biochim Biophys Acta* **999**, 281–288.
28. Pedersen, J., Otzen, D. & Kristensen, P. (2002) Directed evolution of barnase stability using proteolytic selection. *J Mol Biol* **323**, 115–123.
29. Chu, R., Takei, J., Knowlton, J. *et al*. (2002) Redesign of a four-helix bundle protein by phage display coupled with proteolysis and structural characterization by NMR and X-ray crystallography. *J Mol Biol* **323**, 253–262.
30. Van den Burg, B., Vriend, G., Veltman, O.R., Venema, G. & Eijsink, V.G. (1998) Engineering an enzyme to resist boiling. *Proc Natl Acad Sci USA* **95**, 2056–2060.
31. Jaenicke, R. (2000) Do ultrastable proteins from hyperthermophiles have high or low conformational rigidity? *Proc Natl Acad Sci USA* **97**, 2962–2964.
32. Hernandez, G., Jenney, F.E., Adams, M.W. & LeMaster, D.M. (2000) Millisecond time scale conformational flexibility in a hyperthermophile protein at ambient temperature. *Proc Natl Acad Sci USA* **97**, 3166–3170.
33. Fitter, J., Herrmann, R., Hauss, T., Lechner, R.E. & Dencher, N.A. (2001) Dynamical properties of α-amylase in the folded and unfolded state: the role of thermal equilibrium fluctuations for conformational entropy and protein stabilisation. *Physica B* **301**, 1–7.
34. Schuler, B., Kremer, W., Kalbitzer, H.R. & Jaenicke, R. (2002) Role of entropy in protein thermostability: folding kinetics of a hyperthermophilic cold shock protein at high temperatures using (19)F NMR. *Biochemistry* **41**, 11670–11680.
35. Stawiski, E.W., Baucom, A.E., Lohr, S.C. & Gregoret, L.M. (2000) Predicting protein function from structure: unique structural features of proteases. *Proc Natl Acad Sci USA* **97**, 3954–3958.
36. Hubbard, S.J. (1998) The structural aspects of limited proteolysis of native proteins. *Biochim Biophys Acta* **1382**, 191–206.
37. Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zamai, M. & Zambonin, M. (1986) Correlation between sites of limited proteolysis and segmental mobility in thermolysin. *Biochemistry* **25**, 1847–1851.
38. Hubbard, S.J., Beynon, R.J. & Thornton, J.M. (1998) Assessment of conformational parameters as predictors of limited proteolytic sites in native protein structures. *Protein Eng* **11**, 349–359.
39. Fersht, A. (1983) *Enzyme Structure and Mechanism*, 2nd edn. Freeman, W.H. (ed.), pp. 98–120.
40. Duranti, M., Gius, C., Sessa, F. & Vecchio, G. (1999) The saccharide chain of lupin seed conglutin is not responsible for the protection of the native protein from degradation by trypsin, but facilitates the refolding of the acid-treated protein to the resistant conformation. *Eur J Biochem* **230**, 886–891.

41. Darewickz, M., Dziuba, J. & Mioduszewska, H. (1998) Some physico-chemical properties and structural changes of bovine β-casein upon glycation. *Nahrung* **42**, 213–214.
42. Stewart, G.A. & Thompson, P.J. (1996) The biochemistry of common aeroallergens. *Clin Exp Allergy* **26**, 1020–1044.
43. Alberse, R.C. (2000) Structural biology of allergens. *J Allergy Clin Immunol* **106**, 228–238.
44. Simonato, B., Pasini, G., Giannattasio, M., Peruffo, A.D., De Lazzari, F. & Curioni, A. (2001) Food allergy to wheat products: the effect of bread baking and *in vitro* digestion on wheat allergenic proteins. A study with bread dough, crumb, and crust. *J Agric Food Chem* **49**, 5668–5673.
45. van Regenmortel, M.H.V. (1992) Molecular dissection of protein antigens. In: *Structure of Antigens*, Vol. I, Van Regenmortel, M.H.V (ed.). CRC Press, USA, pp. 1–27.
46. Pierson-Mullany, L.K., Jackola, D.R., Blumenthal, M.N. & Rosenberg, A. (2002) Evidence of an affinity threshold for IgE-allergen binding in the percutaneous skin test reaction. *Clin Exp All* **32**, 107–116.
47. Pierson-Mullany, L.K., Jackola, D.R., Blumenthal, M.N. & Rosenberg, A. (2000) Characterization of a polyclonal allergen-specific IgE responses by affinity distributions. *Molec Immunol* **37**, 613–620.
48. Mirza, O., Henriksen, A., Ipsen, H. *et al.* (2000) Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J Immunol* **165**, 331–338.
49. Beardslee, T.A., Zeece, M.G., Sarath, G. & Markwell, J.P. (2000) Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *Int Arch Allergy Immunol* **123**, 299–307.
50. Burks, A.W., Shin, D., Cockrell, G., Stanley, J.S., Helm, R.M. & Bannon, G.A. (1997) Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* **245**, 334–339.
51. Shin, D.S., Compadre, C.M., Maleki, S.M. *et al.* (1998) Biochemical and structural analysis of the IgE-binding sites on Ara h1, an abundant and highly allergenic peanut protein. *J Biol Chem* **273**, 13753–13759.
52. Rabjohn, P., Helm, E.M., Stanley, J.S. *et al.* (1999) Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* **103**, 535–542.
53. Helm, R., Cockrell, G., Connaughton, C. *et al.* (2000) A soybean G2 glycinin allergen. 2. Epitope mapping and three-dimensional modelling. *Int Arch Allergy Immunol* **123**, 213–219.
54. Valenta, R. & Kraft, D. (2001) Recombinant allergen molecules: tools to study effector cell activation. *Immunol Rev* **179**, 119–127.
55. Van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J. *et al.* (2000) β (1,2)-Xylose and α (91,3)-fucose residues have a strong contribution in IgE-binding to plant glycoallergens. *J Biol Chem* **275**, 11451–11458.
56. Alberse, R.C. (1998) Clinical relevance of carbohydrate allergen epitopes. *Allergy* **53** (Suppl 45), 54–57.
57. Dearman, R.J. & Kimber, I. (2001) Determination of protein allergenicity: studies in mice. *Toxicol Lett* **120**, 181–186.
58. Dearman, R.J., Caddick, H., Stone, S., Basketter, D.A. & Kimber, I. (2001) Characterisation of antibody responses to food proteins: influence of route of exposure. *Toxicol* **167**, 217–231.
59. Dunker, A.K., Lawson, J.D., Brown, C.J. *et al.* (2001) Intrinsically disordered protein. *J Mol Graph Model* **19**, 26–59.
60. Brett, G.M., Mills, E.N.C., Bacon, J. *et al.* (2002) Temperature-dependent binding of monoclonal antibodies to C hordein. *Biochim Biophys Acta* **1594**, 17–26.
61. Dyson, H.J. & Wright, P.E. (1995) Antigenic peptides. *FASEB J* **9**, 37–42.
62. Asche, G.L., Lewis, A.J. & Peo, E.R. (1989) Protein digestion in weanling pigs: effect of dietary protein source. *Am Inst Nutr* **119**, 1093–1099.
63. Wong, D.W.S., Camirand, W.M. & Pavlath, A.E. (1996) Structures and functionalities of milk proteins. *Crit Rev Food Sci Nutr* **36** (8), 807–844.
64. Breiteneder, H. & Ebner, C. (2000) Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* **106**, 27–36.
65. Markovic-Housley, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O. & Breiteneder, H. (2003) Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol* **325**, 123–133.
66. Shin, D.H., Lee, J.Y., Hwang, K.Y., Kim, K.K. & Suh, S.W. (1995) High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. *Structure* **3**, 189–199.

# 11 Assessing the Allergenicity of Novel and GM Foods

RENÉ CREVEL and CHARLOTTE MADSEN

## 11.1 Introduction

Assessment of the allergenicity of proteins is a relatively new development in the toxicological assessment of foods and food ingredients. While food allergy has aroused the interest of researchers for many years [1, 2], the focus of the work has been largely on milk and early infant foods [3, 4]. The introduction of novel foods from sources not usually consumed in the country where they are to be marketed (e.g. kiwi fruit in Europe), but more particularly genetically modified (GM) foods, provoked a realisation that allergenicity was a potential hazard that had to be guarded against. Several factors contributed to this realisation. One was the widely reported increase in atopic disease within the industrialised world [5], and a postulated increase in the prevalence and incidence of food allergy. Another was the observation by Nordlee *et al.* [6] that a methionine-rich protein from Brazil nut (*Bertholletia excelsior*), introduced into soy, could bind IgE from Brazil nut-allergic individuals. The authors concluded that if that modified soy were commercialised for animal feed, there was a possibility that it would also enter the human food chain and provoke allergic reactions in Brazil nut-allergic individuals. This incident also highlighted the inadequacy of existing animal models as predictors of protein allergenicity, since the modified soy had been tested in rats and found not to differ from control soy [7]. The realisation that no single predictive test could identify the allergenic potential of an unknown protein led to the formulation of schemes to assess potential allergenicity. The first of these was the IFBC/ILSI decision tree for the assessment of the allergenicity of foods produced by genetic modification [8]. This scheme included assessment of the resistance to simulated gastric digestion [9], as well as amino acid sequence comparison with known allergens, serological tests and human studies. This scheme was subsequently adopted by World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), and then extensively modified and updated in 2001 [10]. Each of these decision trees identifies features of allergens which must be considered and tests which should be performed to give assurance that a novel protein is not likely to sensitise, or elicit reactions. These schemes thus use a weight of evidence approach to assess the likelihood that a novel protein will prove allergenic. Another important date in the history of allergenicity assessment was the formal requirement for such an assessment, enshrined in the European Union's Novel Foods Regulation (97/258EC), promulgated in 1997.

This chapter starts by discussing the issues underlying the need to assess protein allergenicity. It then considers the aims of such an assessment and, in particular, whether an operational distinction can be made between assessment of the risk of sensitisation and assessment of the risk of eliciting a reaction in persons who are

already sensitised. The chapter then reviews the strategies proposed and the methodology available to generate the data on which to make a judgement of potential allergenicity, and current limitations in producing a valid prediction. The chapter concludes with a brief consideration of the methodology which can be used, once the product is on the market, to ascertain whether the judgement is correct.

## 11.2 Food allergy: the issues

### 11.2.1 *What's special about allergenicity?*

Allergenicity and allergy mean different things to different people, both among the lay public, and among the scientific and medical professions. For the purposes of this chapter, allergy is the clinical reaction resulting from the induction of an IgE-mediated immune response to a (food) protein. It thus excludes phenomena such as allergic contact dermatitis provoked by low molecular weight chemicals, and other food-dependent pathologies with immune aetiologies, such as coeliac disease, which involve different immunological mechanisms. Allergenicity is defined as the potential of a (food) protein to induce an IgE-mediated sensitisation, which could subsequently provoke a clinical response.

11.2.1.1 *Structure–activity relationships.* Current knowledge about the relationship between protein structure and allergenicity is not adequate for prediction of protein allergenicity. Aalberse [11] recently reviewed the structural biology of allergens, based on 40 allergenic proteins. He found that they could be grouped into four families based on the secondary structure components (β-sheets, α-helices) they contained, but concluded that allergens possessed no structural features that could distinguish them from non-allergenic proteins. It is possible, however, to use certain characteristics of proteins (e.g. function) to help form a judgement on the probability of it being allergenic, as discussed elsewhere in this book.

11.2.1.2 *Biology of IgE responses.* Food proteins gain rapid access to both the mucosal and the systemic immune systems, but the normal response to such proteins is oral tolerance, which arises after initial sensitisation [12]. While IgE responses are extremely tightly controlled, the biology of such responses offers a challenge to risk assessment in food allergy. It is now generally accepted that repeated low doses of allergen are very effective in producing an IgE-mediated response, while high doses result in tolerance [12], although the precise kinetics of the dose–response relationship remains to be established. It is still not completely clear whether the mechanisms involve active suppression of IgE production, as suggested by the results of Arps *et al.* [13], or a biasing of the response towards a Th2 phenotype, as indicated by Rogers and Croft [14]. Irrespective of the mechanisms involved, another issue is what constitutes a low or high dose in the context of the consumption of a food and subsequent recognition of proteins by the mucosal immune system.

11.2.1.3 *Lowest eliciting doses (thresholds of reactivity).* The biology of IgE responses impacts principally on how IgE-mediated sensitisation can be avoided, but for existing

plant proteins a critical consideration is the amount of allergen, which is capable of provoking a reaction in susceptible (sensitised) individuals. Only limited data have been published on thresholds [15], and often they do not lend themselves readily to risk assessment as results have been established using different protocols. Currently, modelling of population responses, which is required to determine safe levels of allergen, is inadequate. What the existing data do indicate is that the amounts can be very small and vary considerably from one allergic person to the next [16,17].

11.2.1.4 *Epidemiology of food allergy*. Food allergy is classed as an atopic disease, on the basis that it involves IgE-mediated responses and that individuals who are atopic are more predisposed to develop food allergies than non-atopics. Indeed it has been proposed that sensitisation to foods in early childhood is an early indicator of the likely subsequent development of inhalant allergies (*the allergic march*) [18]. Atopic diseases have increased considerably in prevalence and incidence over the last 20 to 30 years at least, and the obvious concern is whether food allergy has followed the same trend or will do so in the future. Historical data on prevalence do not span a sufficient time yet, to be able to draw any conclusions, but the burden of food allergy at approximately 1–2% of the overall population and possibly 5–8% of children in the industrialised world is already significant [reviewed in 19]. Moreover, the public perception of the prevalence of food allergy is even higher, at approximately 20% [20], with a resulting pressure on those responsible for public health to act to reduce it.

### 11.2.2 *Why is allergenicity a special concern?*

Allergenicity has been singled out as a toxicological endpoint which must be specifically addressed in the EU Novel Foods Regulation (258/97/EC) [21]. Other regulatory bodies have similarly marked allergenicity for special consideration (FAO/WHO, Codex Alimentarius Commission), both in assuring the safety of conventional foods and for approval of novel foods, particularly those of GM origin. Several reasons for this situation exist:

- Proteins are the main constituents of foods responsible for allergic reactions. It therefore follows that a novel protein introduced into the food supply may prove to be an allergen. The need for an assessment of this risk is sharpened by inadequate knowledge at the molecular level of the relationship between protein structure and allergenicity, as well as the absence of adequate predictive tests. The consequence is that all proteins for which there is no history of exposure, whether present in GM crops or in novel foods, must be considered potentially allergenic for purposes of safety assessment.
- Food allergy is perceived as an important public health issue, particularly given the rising prevalence of allergic disease. The impact of food allergy on the quality of life of sufferers is also a significant factor.
- Allergic reactions to food can be severe or life-threatening in a small number of cases, and there is a public expectation that regulatory measures will exist to minimise the likelihood of such reactions. The absence of such measures would diminish public confidence in food safety.

### 11.2.3 *Allergenicity and GM crops and foods*

A major aspect of the genetic modification of crops is the insertion of a gene coding for a protein with an attribute desirable in the recipient crop, as in the insertion of the Brazil nut 2S albumin gene to improve the nutritional quality of soy [7], or the insertion of genes for the Bt crystal protein into a variety of crops to confer resistance to insect pests [22]. Because any protein, other than one known from a history of use not to be allergenic, has to be considered *a priori* a potential allergen, as discussed above, safety assessment must clearly include an evaluation of allergenicity. However, other types of genetic modification may also require that the resulting crop, or crop component, be assessed for altered allergenicity. Specifically, evidence will be required that existing allergens in the crop are not up-regulated and that there is no (increased) synthesis of bio-active materials, which could facilitate sensitisation, such as saponins which are used to boost the response in an experimental animal model of food allergy [23].

### 11.2.4 *Allergenicity and novel foods (other than GM)*

Regulation 258/97/EC [21] recognises several classes of novel foods apart from those produced by biotechnological methods. Among others, these include foods which have not been commonly consumed in the EU and those which have been produced using novel processes. An assessment of potential allergenicity is also required for these foods. The approach to this assessment would need to be tailored to the novel food in question, based on scientific knowledge about how allergenicity might be altered. In the case of a new plant food, relevant considerations would be the allergenicity of the plant food that it would match most closely, the likelihood of it cross-reacting with plant foods currently consumed and whether it would it be more allergenic than the plant food(s) it would displace (if any). In the case of a food produced through a novel process, the main question would be whether the process would alter the activity of any existing allergenic components or would create neo-allergens.

## 11.3 Allergenicity assessment

### 11.3.1 *Aims*

An assessment of allergenicity should ideally produce data which can be used to prepare a complete risk assessment. These data should include information on:

- How likely is the protein of interest to sensitise susceptible individuals, if they consume it?
- If someone becomes sensitised, what is the likely lower eliciting dose (threshold)?
- Is the protein likely to produce reactions in individuals allergic to the source of the protein?
- What is the likelihood of producing a reaction in someone already sensitised to a different food (cross-reactivity)?

At present, the tools available can only give partial answers to these questions.

### 11.3.2 *Strategies*

The first systematic approach to assess the allergenic risk from GM-food [8] suggested a decision tree based on direct and indirect comparison of allergen structure and evaluation of stability to digestion and processing. This first decision tree (Fig. 11.1) greatly influenced subsequent discussions.

11.3.2.1 *European Community.* According to EU regulations [21, 24], evaluating the allergenicity of a novel food from a GM source should include consideration of the allergenic potential of the donor and of the recipient organism. *In vitro* and *in vivo* tests in individuals allergic to the traditional food counterpart are suggested to this end, although it is recognised that this approach raises ethical problems. If the novel protein comes from a source that is known to be an allergenic food, specific immunological tests using sera from allergic individuals are suggested. If these tests are negative, *in vivo* skin prick tests and oral challenges may be performed. The regulations also recommend the evaluation of possible indicators of allergenicity, such as epitope sequence similarity with known allergens, heat stability, sensitivity to pH, resistance to hydrolysis by gastrointestinal proteases, plasma levels and molecular weight. Human data from pre-marketing trials, together with reports of sensitisation in workers, were also recommended as sources of additional evidence. As with other toxicological endpoints, the choice of tests should be made on a case-by-case basis, guided by scientific feasibility, necessity and the interpretability of the results. The regulation also

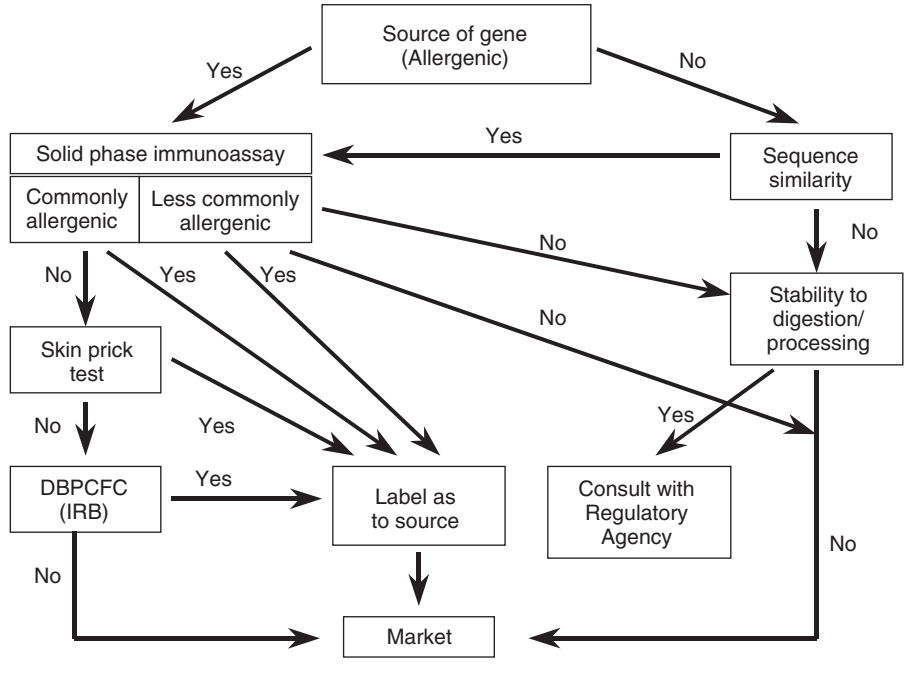


**Fig. 11.1** IFBC/ILSI 1996 decision tree for assessing the allergenicity of foods produced from genetically engineered crop plants (taken from [9]).

acknowledged that new approaches were needed to assess the potential allergenicity of novel foods in humans.

11.3.2.2 *WHO/FAO*. The FAO/WHO Expert Consultation on Foods Derived from Biotechnology proposed a new decision tree (Fig. 11.2) for assessing the likely allergenicity of novel proteins. It updates the original decision tree (essentially the same as in ref 8) and proposes some interesting new ideas [10], namely different decision pathways and elimination of any requirement for *in vivo* human testing. It also recognises that animal models may be of value, although it recognises that no validated ones are currently available. Another notable innovation is that it describes, in some detail, many of the procedures to be used and how some of the findings may be interpreted. In doing so, it seeks to encourage the adoption of harmonised methodology which would not only make evaluations easier to compare and reproduce, but also permit an eventual assessment of their predictive value. Key features of the new recommendations include an outline description of the amino acid sequence comparison, a detailed protocol for the measurement of pepsin resistance and new guidance on serum screening procedures to improve the significance of results. In the new scheme, sequence similarity is accorded an enhanced role. The consultation report suggests that cross-reactivity between the expressed protein and a known allergen (as can be found in the protein databases) has to be considered when there is either:

1. more than 35% identity in the amino acid sequence of the expressed protein (i.e. without the leader sequence, if any), using a window of 80 amino acids and

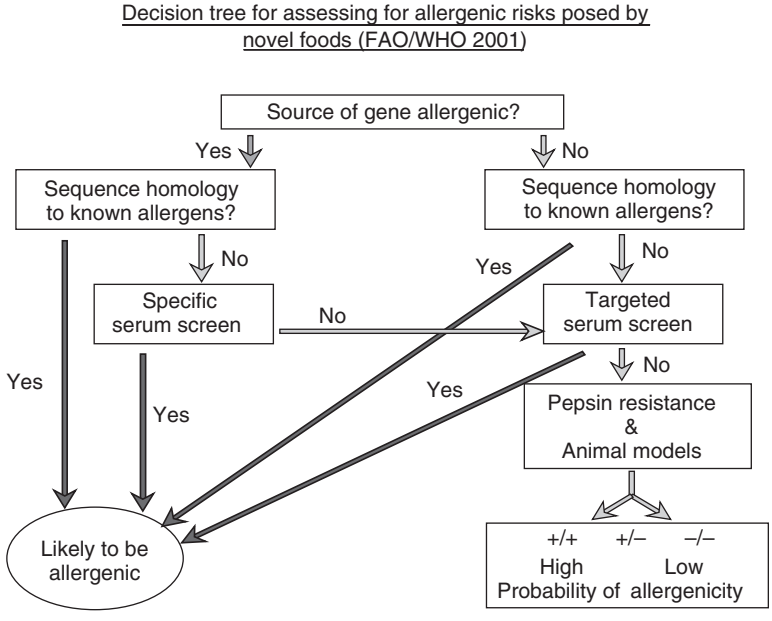


**Fig. 11.2** FAO/WHO (2001) decision tree for assessing the allergenicity of products of modern biotechnology (taken from [10]).

a suitable gap penalty (using Clustal-type alignment programs or equivalent alignment programs); or
2. identity of six contiguous amino acids.

Since an identity of six contiguous amino acids between an allergen and a given protein sequence has a high probability of occurring by chance, verification of potential cross-reactivity is warranted when criterion (1) is negative, but criterion (2) is positive. In this situation, suitable antibodies (from human or animal source) have to be tested to substantiate the potential for cross-reactivity. Alternatively, if a protein has an identity score with a known allergen that equals or exceeds 35%, the protein can be considered to be a likely allergen and no further testing is suggested. This conclusion is based on the knowledge that families of allergens where there is clinically significant cross-reactivity and high sequence similarity have been described. For instance, the major apple allergen (Mal d 1) has sequence homology with the major birch pollen allergen (Bet v 1) of 56% and with the major hazelnut pollen allergen of 54% [25]. However, a high degree of sequence similarity may also occur without clinically relevant cross-reactivity. For example, tropomyosin from chicken has 60% sequence homology with tropomyosin from shrimp, the major shrimp allergen, but there is no IgE cross-reactivity between shellfish and vertebrate tropomyosins [26]. As there are no generally accepted criteria for defining significant matches, sequence alignment must be combined with other considerations such as the source of the protein, stability to digestion and stability to processing [27].

If there is no sequence similarity between the novel protein and known allergens, the recommendation from the FAO/WHO consultation is that the protein should be tested against serum from patients. This can take place either in a specific serum screen, if the protein is from an allergenic source, or in a targeted screen if the sera for a specific screen are not available, or available in insufficient numbers to produce data with the desired level of statistical significance. The targeted screen is also proposed in the event of the specific serum screen being negative. The concept of the targeted serum screen is to test the protein against sera from people allergic to broadly related species. Thus, if the recombinant protein is derived from a monocotyledonous plant source, it is proposed to test serum samples from patients with high levels of IgE antibodies to monocot allergens such as grass and rice. Similarly, with a recombinant protein derived from a dicotyledonous plant, serum samples from patients with high levels of IgE antibodies to dicot allergens such as tree pollen, weed pollen, celery, peanuts, tree nuts and latex should be used. A similar approach is suggested if the recombinant protein is derived from a mould (fungus), an invertebrate or a vertebrate. Such a screen should include 25 individual serum samples with high levels of IgE to the selected group of airborne allergens and (if applicable) 25 serum samples with IgE to the selected group of food allergens.

This targeted serum screen will determine whether the novel protein has IgE epitopes identical to those present in putatively related inhalant or food allergens. This approach is pertinent, as a number of food allergies are caused by cross-reaction to inhalant allergens. However, with our current lack of knowledge regarding the mechanisms of food allergenicity, the positive predictive ability of the targeted serum screen is not known, making a risk assessment difficult. As a consequence of this lack

of knowledge, it has been suggested that if a protein is negative in the targeted serum screen, its pepsin resistance should be evaluated. In addition, it should be tested in an animal model, although there are currently no well-validated animal models available. Further evaluation tests in humans, such as skin prick tests and oral provocation, may also be needed. These are mentioned in the WHO report but are not included in the decision tree, based on the acceptance that they may not be performed for ethical reasons.

To summarise, the main innovations in the FAO/WHO decision tree are:

1. defining how analysis of sequence similarity should be performed and interpreted;
2. introducing targeted serum screen where no sera exist to the source of the protein;
3. suggesting the use of animal models.

11.3.2.3 *Codex Alimentarius.* Because the FAO/WHO report introduced a new approach to the assessment of allergenicity, the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology decided that an Annex, containing detailed procedures for the allergenicity testing, should be prepared. An *Ad Hoc* Open-ended Working Group was formed to draft such an Annex.

The *Ad Hoc* Open-ended Working Group declares in the introduction that an integrated, stepwise, case-by-case approach should be used to assess the possible allergenicity of newly expressed proteins [28]. As in the FAO/WHO tree, the first step should be comparison of sequence similarity. The group is aware that searches for six identical amino acid searches may increase the likelihood of identifying false positives. Pepsin resistance should also be included in this first step. A positive sequence similarity or a positive result in a specific serum screen suggests that the protein is a likely allergen, and further development should be discontinued. If the source of the gene is not allergenic and no sequence similarity with known allergens is found, the protein is considered not to be a likely allergen and also unlikely to be cross-reactive to known allergens. These results should be interpreted together with other data. Targeted serum screening and animal models are not included but mentioned under areas requiring further development. This Annex is now under discussion in the Codex system (step 3 March 2002).

Before targeted serum screening and animal models are more developed and validated, results from these tests may be difficult to interpret and therefore to use in hazard identification. At present, if a new protein comes from a non-allergenic source (the right side of the decision tree), the possibility for hazard identification is limited to sequence homology and resistance to degradation. There is therefore an urgent need to develop new test methods and testing strategies.

## 11.4 Methods for assessment of allergenicity

### 11.4.1 *In silico methods*

11.4.1.1 *Sequence analysis.* All formal schemes for the assessment of potential allergenicity, including the latest FAO/WHO decision tree, incorporate an element of comparison of the primary structure of the protein with that of known allergens [8, 10].

IgE (and other antibodies) recognises structures on proteins, to which they bind. These structures all ultimately derive from the primary amino acid sequence of the protein, but may belong either to the primary structure (linear epitopes) or to the secondary or, more usually to the tertiary structure (conformational epitopes). Analysis of sequence similarity can provide assurance that a novel protein does not share significant lengths of primary sequence, and therefore linear epitopes, with known allergens. This helps to provide assurance that the protein of interest will not bind to antibodies to known allergens, present in individuals sensitised to them. Sequence analysis can thus help to establish whether the protein is likely to prove unsafe for existing allergic individuals. It can also provide other information by identifying possible similarities with protein classes which contain known allergens (e.g. 2S albumins, pathogenesis-related proteins).

11.4.1.2 *Methodology.* Sequence analysis uses public domain databases, containing either the primary sequence of proteins or the nucleotide sequences from which such protein sequences can be derived. These were first established in the 1960s, initially on paper and later in electronic form, and have grown exponentially since that time. These databases, and their associated tools, were designed primarily for the study of protein structure, function and evolution, rather than for identification of potential allergenic epitopes in novel proteins. The consequences with regard to their value and role in allergenicity assessment were discussed extensively by Gendel [27].

11.4.1.3 *Databases.* Sequence databases comprise nucleotide and protein sequence databases. Historically, the protein sequence databases preceded the nucleotide ones. However, as a result of genome sequencing and similar activities, the nucleotide sequence databases have now far outgrown the protein ones. Most protein sequence information therefore originates from translation of nucleic acid sequences, and several protein sequence analysis programs use this information directly.

Three primary nucleotide sequence databases exist: GenBank (run by the National Center for Biotechnology Information – NCBI, USA), EMBL (run by the European Bioinformatics Institute, Europe) and the DNA Database of Japan (DDBJ). These nucleotide sequence databases contain sequences submitted directly by researchers. The role of the DNA database is to collect all these sequences, store them, curate them and make them available in a consistent format to the global scientific community. All these databases participate in the International Nucleotide Sequence Databases Collaboration (INSDC). In practice, this means that these databases exchange data daily, although they retain individual features such as accession numbers. Sequences need therefore to be submitted to one database for them to be included in all of them, and searches in each database will be similarly effective. In this collaboration, only the database to which a sequence has been submitted can update that particular entry. These DNA databases can all be considered primary databases in as much as they receive data, which have been generated experimentally in a laboratory, rather than translated from some other sequence or generated by some theoretical model. The mission of the DNA database is neither to select data which are valid, nor to present a critically edited version of the data. A consequence of this is that the database may contain different copies of the same sequence, as well as overlapping sequences.

Protein sequence databases are generally secondary databases, with very few sequences being determined by direct protein analysis. Instead, most of the information they contain is derived from the nucleotide sequence databases by translation. About 95% of the sequence data in SWISS-PROT, for example, come from the translation of DNA sequences available in the nucleotide sequence database (GenBank/EMBL/DDBJ). The protein sequences in SWISS-PROT are thus almost exclusively derived from the data stored in the DNA archive. The added value of SWISS-PROT and other protein sequence databases resides in the high level of annotations (such as the description of the function of a protein, its domain structure, post-translational modifications, variants, etc.), the minimal level of redundancy and the high level of integration with other databases. This work obviously requires making judgements on the validity of some results. TrEMBL is a computer-annotated supplement of SWISS-PROT that contains all the translations of EMBL nucleotide sequence entries, not yet integrated in SWISS-PROT. On 5 March 2002, Swiss-Prot contained 105 967 entries, and TrEMBL 594 148 entries. For comparison, a BLAST search for non-redundant GenBank CDS translations PDB+SwissProt+PIR+PRF gave 419 232 sequences.

Protein and nucleotide sequence databases are part of a community of databases, which are co-managed [29]. This provides ways for the data contained to be checked. SWISS-PROT, TREMBL, the Protein Information Resource (PIR) and the Protein Database (PDB) are all part of this community. Because of the co-operative agreements under which the nucleotide databases operate, sequences can be retrieved from any of the general databases, although the information provided with the sequence will differ. Other secondary databases also exist, but they are restricted to particular types of protein for each database (e.g. MHC molecules, yeast proteins, etc.).

The information stored in the nucleotide and protein databases differs because of their contrasting roles. In addition to the sequence data, the nucleotide databases contain taxonomic and bibliographic information as well as descriptors, which uniquely identify a sequence and link it to related ones. There is little interpretative information. In contrast, protein databases, of which SWISS-PROT is perhaps the best model, contain, in addition to the sequence and identifying descriptors, information such as the description of the function of a protein, its domains' structure, post-translational modifications and variants.

11.4.1.4 *Comparing sequences and determining similarity.* Sequence similarity between proteins can be established for the whole protein (global alignment) or for sequences within the proteins (local alignment). Since recognition of proteins by T-cell receptors or antibody-binding sites only involves the relatively small sequences that form the epitopes, local alignment is logically the most relevant. However, it is also useful to know whether an unknown protein shares a significant proportion of its sequence with an allergen, since individual epitopes are not defined for most proteins. Moreover, this information can indicate whether the protein of interest belongs to a family which contains known allergens. Several algorithms have been proposed, but the most frequently used are FASTA [30] and BLAST [31], from which computer programs of the same name have been generated. Both methods rely on assessing the probability that an alignment between a query sequence (the unknown protein) and a sequence in the database occurs by chance.

The FASTA algorithm of Pearson and Lipman [30] is the most frequently used method for global alignments. The current version offered by databases and search interfaces is FASTA3, which is one of a family of related programs, which differ in the sort of sequence they are designed to compare. The principle used to determine the degree of global similarity is to compare the protein of interest with those in the database, using pairwise comparison of amino acids. These comparisons are done for segments of specified word lengths (i.e. amino acid number) and to generate segments with several matches along the protein. The segments are given scores, which are a function of the number of successful matches, with negative scores for gaps and non-matching amino acids. The program also uses a substitution matrix, that is a table of scores for mismatched amino acids at particular points in the sequence. This type of matrix allows conservative substitutions to attract a lower penalty, than those in which there is a complete change in the type of amino acid. The initial segments are then further combined and scored. Finally, the program finds an optimised gapped alignment around the initial segment which gave the best score. The results include an estimate of the likelihood of particular alignments arising by chance.

The program automatically searches for and eliminates regions of low complexity, for example multiple repeats of one or two amino acids, which would otherwise result in apparently significant homology, but without necessarily having any biological significance.

Some of the limitations of global alignment include the fact that the statistical basis for the procedure is not fully established, since the shape of the distribution of alignments within the database is not known. As a result, any probability estimate is approximate. Another limitation is that the scoring matrices and, therefore, the scores given for mismatched amino acids and gaps are arbitrary, although different matrices can be used. No matrices exist which address the effects of specific amino acid substitutions on protein binding to antibodies or T-cell receptors.

BLAST (Basic Local Alignment Search Tool) is the most commonly used algorithm for establishing local alignments between protein sequences. Unlike FASTA, it has a firm statistical basis, using the methods of Karlin and Altschul [31]. The BLAST program works on the basis of finding High Scoring Segment Pairs (HSPs), which are pairs of sequences of equal length (one in the query protein, the other in the database protein) whose scores cannot be improved by extension or trimming. The current version (2.0) allows gaps in local alignments and imperfect matches, using a substitution matrix to score non-matching amino acids. The expected number (E) of HSPs with a score of at least S is calculated for each match, and is a measure of the probability of such a match occurring at random in the searched database. The E-value is a selectable threshold for reporting matches, so that distant similarities can be identified, if appropriate. As for the FASTA program, low complexity regions, which would be expected to give very high alignment scores without biological significance are screened out. The limitations of FASTA, with respect to the substitution matrices, apply equally to BLAST analyses.

11.4.1.5 *Limitations of protein sequence similarity analysis.* Protein sequence databases contain data about the primary sequence of proteins. Aside from specific limitations pertaining to the tools used to analyse sequences, a major limitation with respect to their ability to identify potential allergenic epitopes is that many of the latter are

conformational rather than sequential. The use of 3D-protein sequence databases offers a possible solution, but the number of proteins in such databases is at present too small to be of value. The search term *allergen* gave 1869 hits through the Entrez interface for protein databases (March 2002), but often more than one sequence has been submitted for each allergen (e.g. corresponding to a partial sequence or an isoform). The number of individual known allergenic proteins is therefore much less than 1869. In contrast, the number of sequences retrieved by the same search term in the Brookhaven Protein Databank (PDB), which stores 3D-proteins structures, was 42 out of 17 869 structures, many entries being for the same molecule under different conditions of crystallisation. Another limitation is that the allergenic proteins in many foods have not yet been identified, and therefore not sequenced and submitted to databases.

Some limitations pertain to the software itself and its use. The implementation of all search algorithms requires various compromises in order that the searches can take place within a reasonable time.

Allergenic epitopes recognised by antibodies are generally about eight amino acids long, although lengths of linked shorter multiple repeats can occur (e.g. $2 \times 4$ amino acids separated by two other, non-binding amino acids) [32]. However, for any given size of database, the likelihood that a query sequence will match a sequence in the database by chance increases, as the length of the sequence diminishes. In practice, this means that because such matches have a high probability, they will not be reported, even though they may be relevant. It may be possible to overcome this problem partially by only searching against a subset of all sequences, e.g. allergens, provided that such a subset can be generated. It is also possible to adjust search parameters, such as the threshold E-value above which matches should not be reported, or to use different scoring matrices, which for example introduce larger gap penalties. However, this can often result in large numbers of irrelevant matches, which have to be sorted through to identify potentially relevant hits [33]. Another limitation of the tools available for analysing protein sequences is that most of the tools have been optimised for identifying sequence similarity, in order to classify an unknown protein as a function of the family to which it belongs and the function which it performs. Such parameters can be useful in the context of allergenicity assessment, but at the amino acid sequence level, exact matches with relatively short sequences are more appropriate. Programs such as Peptide Match, available in the PIR database, can also help in identifying matches [34].

The FAO/WHO consultation (2001) was the first to recommend a specific approach to sequence comparison. It is briefly as follows. Firstly, all polypeptides that can be generated using an 80-amino acid reading frame should be generated from the protein of interest. These polypeptides should then be compared with a database containing all known allergens. Based on knowledge of cross-reactivity and protein structure, a 35% or greater similarity with known allergens then triggers a serological examination of the protein, using sera containing antibodies to the putative-related allergen. Should no global similarity be established, all possible hexamers should be generated from the novel protein sequence (excluding any leader sequences and any other elements not present in the final translated product). These hexamers should then be compared with the proteins in the same allergen database, looking for exact matches, if possible. The six-amino acid reading frame was proposed as a more sensitive

replacement of the original eight-amino acid frame of the IFBC/ILSI scheme [8], which was based on the minimum number of amino acids constituting an IgE antibody epitope. The logic for this choice was based on the observation that in some instances, matches of as few as four amino acids [32] were immunologically significant, although only in the context of a hexadecapeptide in which two of those sequences were separated by an irrelevant hexamer. Additionally, some of the regions responsible for IgE binding in the peanut allergens Ara h 1 [35] and Ara h 2 [36] were also found to consist of a minimum of six contiguous amino acids, although most were eight to ten amino acid long. Applying these principles to an ice-structuring protein from fish [33, 37], we found that reducing the reading frame to exact matches of seven amino acids produced six hits with unrelated proteins in the PIR database, while using six amino acids generated 515 matches, none of which were biologically significant. Thus, while the aim of the proposal, i.e. to make sequence analysis more predictive, is commendable, there is a need to establish the general validity and practicability of such recommendations. Indeed, the briefing paper concluded that a match of at least eight contiguous identical amino acids of the protein in question with a known allergen was useful for identifying potentially allergenic components [38]. Given the limitations of what such an analysis can yield, it is questionable whether it is of value to direct efforts to improve it in that direction, rather than towards the development of publically available allergen databases and specific tools for sequence analysis within those databases.

### 11.4.2 *Immunochemical methods*

*In silico* methods can help predict the allergenicity of unknown proteins through identification of the class of protein to which they belong and their possible function. At a molecular level, they can identify structures which may bind IgE and which constitute warnings, not merely of the sensitising potential of the protein, but also of the potential to provoke reactions in individuals already sensitised to another protein possessing the same structures. However, as discussed above, such methods do not at present provide conclusive evidence of hazard. Conversely, the absence of similarity to known allergens cannot be taken to exclude entirely the possibility of cross-reactivity. In particular, where a protein is derived from a known allergenic source, it is prudent to establish that it will not pose a risk to individuals already sensitised to the source of the protein. For instance, if a minor protein from peanut is used, then it is essential to confirm that it is not recognised by IgE antibodies from a selection of peanut-allergic individuals. *In vitro* immunochemical studies of IgE binding can provide such information. Methodologies used fall into two categories:

1. Those which are essentially chemical in nature and which measure IgE binding alone. Prototypes of these assays include the radioallergosorbent test (RAST) and its many variants, including inhibition RAST and equivalent assays with non-radioisotopic readouts. Numerous variants of the different methods exist and the reader is referred to them for details of experimental methodology [39, 40]. Another widely used methodology is immunoblotting (western blotting), which helps to identify the individual proteins which bind IgE. Basically, a mixture containing the

protein of interest is separated on a gel on the basis of molecular weight using sodium docecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins are then electroblotted onto a suitable membrane (e.g. nitrocellulose or PVDF), after which they are incubated with serum containing IgE antibodies of interest in order to identify binding. Binding can be quantified using densitometry and appropriate standards. Some of the drawbacks of immunoblotting include the fact that glycosylated proteins do not run in accordance with their molecular weights. Also, labile proteins need to be handled with care to emerge immunochemically intact from the harsh procedures of SDS-PAGE.

2. Those that not only give an indication of IgE binding but also provide information on whether this binding is biologically meaningful. These methods are cell-based and mimic the biological events which take place when allergen binds to specific IgE bound to effector cells, such as basophils or mast cells. Variants of the assays exist, based on both human and animal cells [41, 42]. In essence, the assays are as follows. Cells are either passively sensitised with IgE of the appropriate specificity, or are obtained from patients whose serum contains the appropriate IgE (and therefore are coated with this IgE). The cells are then treated with the protein or protein mixture of interest and mediator release is measured in response to this stimulus, indicating whether any IgE binding observed in RAST or immunoblotting assays is biologically significant.

### 11.4.3 *Pepsin stability*

An assessment of resistance to pepsin was included in the original IFBC/ILSI decision tree [8]. The reasoning behind this inclusion was that in order for a protein to induce an immune response, it needed to be *seen* by the immune system in a reasonably intact condition. Proteins able to resist degradation under the conditions prevailing in the stomach would therefore be more likely to be allergens than those which were degraded rapidly. Astwood and Fuchs [9], using the protocol listed in the US Pharmacopea [43], provided experimental evidence to support this hypothesis. Thus they demonstrated that Rubisco (ribulose bis-phosphate carboxylase/oxygenase), which has never been shown to produce an IgE response, was rapidly degraded (<15 seconds), whereas allergens such as Ara h 2, ovalbumin and β-lactoglobulin, for example, were largely intact after 60 minutes exposure to simulated gastric juice. Other experimental evidence to support a reduction of allergenic potential by proteolysis comes from vast experience with cow's milk protein hydrolysates, which are prepared for infants who suffer from cow's milk allergy. Findings indicate that allergenic potential starts to decrease substantially when the fragments fall below 6 kDa in molecular weight, and that where the fragments are below 2 kDa, there is no risk of precipitating reactions [42]. However, there are numerous exceptions to the rule that rapidly degraded proteins present little or no allergenic risk. For instance, bovine serum albumin (BSA), which breaks down in less than 15 seconds [9, 33], has been shown to be one of the allergenic components of cow's milk, even though it is only present in small amounts [44]. The hypothesis that largely intact proteins are required to stimulate an immune response has also been questioned on the basis that proteins such as BSA evoke

an IgG-mediated response when given orally, even when they do not produce an IgE-mediated response [45]. The protocol used has also been criticised because it is a poor mimic of true degradability, as it does not take into account critical aspects such as the food matrix. Moreover, the protocols used have not been standardised, and relatively small changes in some parameters can radically alter the outcome of the test [46]. For example, the insecticidal Bt protein Cry 9c from *Bacillus thuringiensis* is stable in gastric juice at pH 2.0, but is labile at pH 1.2 and 1.5 [46]. The FAO/WHO (2001) consultation attempted to address these issues by recommending a standardised protocol, adherence to which would permit much easier comparison of data. It also recommended the use of the term *pepsin resistance* instead of *simulated digestion* so that it would be explicit that it was not a mimic of the gastric digestion process. The recommended procedure consists essentially of incubating the protein of interest (500 µg in 200 µl) in a solution of pepsin (0.32%) maintained at pH 2.0 at a temperature of 60 °C for up to 60 minutes in a shaking water bath. Aliquots should be sampled at 0, 15 and 30 seconds, and 1, 2, 4, 8, 15 and 60 minutes. SDS-PAGE (under reducing and non-reducing conditions) was recommended for assessment of degradation, together with immunoblotting to identify the potential IgE-binding capacity of any fragments, if possible. The use of a standardised protocol will provide a valuable benchmark against which to assess protein degradability, but recent experience indicates that a variety of methods for assessment of degradation should be considered [33], as SDS-PAGE is less than optimal for the detection of glycoproteins and very small fragments. Additional methods available to assess breakdown and characterise fragments include High Performance Liquid Chromatography (HPLC), Gel Permeation Chromatography (GPC) and Matrix-assisted Laser Desorption Ionisation–Time of Flight (MALDI–TOF) mass spectrometry.

In addition to the protocol recommended by the FAO/WHO consultation, a more complex gastrointestinal model (TIM) has been developed that simulates to a high degree the physiology of the stomach and small intestine of monogastric animals and man [47]. However, this model has not been extensively examined in the context of protein allergens.

### 11.4.4 *Animal models*

An animal model with the capacity to predict the allergenicity of food proteins would be very helpful in hazard assessment of novel foods. Several groups are working with mice or rats to achieve this using different approaches. Some researchers consider that it is important that the model should be able to predict the allergenicity of a protein via the oral route, while others consider that the model should predict whether the protein is able to induce an IgE response, irrespective of the route of exposure. Some have used adjuvant to overcome problems with oral tolerance, although adjuvant may skew the immune response. For the time being, the ideal animal model for food allergy does not exist and there is no consensus on whether it should be able to predict the allergenicity of a specific food (i.e. including potential matrix effects) or only the inherent allergenic properties of the protein of interest.

If animals are to be used to predict allergenicity, it is important that their immune response identifies the same allergenic structures (i.e. epitopes) as humans. Limited

data that are currently available suggest that they do. Studies in the Brown Norway rat have demonstrated that the rats identify the same proteins and protein structures in milk and egg as humans [48, 49]. IgE from C3H/HeJ mice recognises the same peanut allergen isoforms and epitopes, as IgE from human peanut-allergic patients [50]. An important technical detail when studying protein allergenicity is to ensure that the animals have been on a diet, free from the protein under investigation, for at least two generations prior to testing [51].

One of the problems when developing a model for food allergy is that the normal physiological response to ingestion of soluble foreign protein is development of oral tolerance. Hence, a model has to change this normal response. Animal models have been developed to study the mechanisms of food allergy. Here, cholera toxin [52] or $Al(OH)_3$ [53] has been used to help inducing a specific IgE response. However, these models were not developed to study the allergenicity of unknown proteins with a view to assessing the potential risk due to exposure.

In contrast, Atkinson and colleagues [54] developed a model specifically designed to predict protein allergenicity, which used the sulphated polysaccharide carrageenan as an adjuvant. In this model, Brown Norway rats are injected intraperitoneally (i.p.) with protein (0.01–1000 µg), together with carrageenan as adjuvant. Antigen-specific reaginic antibody (IgE) was measured by passive cutaneous anaphylaxis (PCA), a semiquantitative method, at day 28. Dose response curves depicting the frequency of animals producing an IgE response against dose of protein required to produce 50% response (ED50) can be read off the graph. The ED50 provides a measure of the relative allergenicity of the proteins. Using this method, the investigators found the following order of allergenicity: lactoferrin > ovalbumin grade II > chicken egg white cystatin > ovalbumin grade VII > bovine serum albumin. The ED50s for lactoferrin and bovine serum albumin were 40–50 ng and 10 µg respectively. Results from unknown proteins can be ranked with known allergens, and the potential allergenicity may be predicted.

One of the weaknesses of this model is that the protein and adjuvant are injected i.p., together. In an alternative model, the authors [55] injected carrageenan i.p. as adjuvant, while giving protein orally as a single bolus dose (gavage) twice a week for six weeks, with and without saponin. Using this model, the allergens were ranked in order of potency as ovalbumin grade II > lactoferrin > bovine serum albumin [56].

Brown Norway rats have also been used in an oral model, without the use of adjuvant. In this model, young male rats are dosed by gavage with 1 mg ovalbumin/rat/day for 42 days. Serum samples are analysed for specific IgG and IgE using ELISA. In this experiment, seven out of eight animals developed specific IgG and IgE. Because the response is measured by ELISA, both the number of animals responding and the magnitude of the response can be used as a measure of potential allergenicity [49].

Other researchers have used the same protocol to sensitise Brown Norway rats, but were unable to induce a convincing specific IgE response [44, 57]. In our hands, specific IgE is induced in 25–60% of Brown Norway rats, after gavage dosing with ovalbumin or egg white (Madsen, unpublished). Brown Norway rats are inbred and *sub strains* of Brown Norway rats with different capacities for IgE production may exist. It may also be difficult to ensure that rats obtained from commercial breeders have not been exposed at some stage of their life to the protein of interest.

High IgE responder BALB/c mice have also been proposed as a model and used to compare the influence of route of exposure on response. IgG and IgE responses after two intraperitoneal injections of 0.5 mg peanut agglutinin, 5 mg ovalbumin or 25 mg potato protein extract were compared with the IgG and IgE responses after gavage dosing with 1 mg/day of the same proteins for 42 days. Both intraperitoneal and gavage dosing resulted in an IgG response. Peanut agglutinin produced an IgE response in all animals, irrespective of route. Ovalbumin induced IgE in only two out of six animals after gavage but in five out of five animals after intraperitoneal administration. Potato protein extract induced an IgE response in two out of six animals after gavage dosing, and in three out of five i.p. administered animals, but titres were very low. In analyses of pooled serum samples, it is evident that intraperitoneal injections of peanut agglutinin or ovalbumin induce the strongest IgE response compared to gavage. The IgE response to potato protein extract is weak both in i.p. administered and gavage dosed animals [44]. At present, the intraperitoneal route therefore looks promising, given that it appears to overcome some of the problems of the oral route, while inducing the same response qualitatively. Clearly, if early results are confirmed with a more extensive range of proteins, this model could prove valuable in hazard characterisation. However, full risk assessment would require a system, which can take account of such factors as fate in the gastrointestinal tract.

At present, there are very few, if any, data on exposure by the oral route, which can be interpreted to assess the probability of sensitisation. Animal models can be used to rank proteins on the basis of their relative allergenic potency, and the applicability of this ranking to man can be assessed by comparison with the rank order of potency in man, as established by a combination of epidemiological and exposure data. Once validated in this way, an animal model could provide information on the allergenic potency of a novel protein. However, data generated in animals can only be extrapolated to man with caution, given that most of such data have been generated in inbred strains of mouse or rat. Such strains have distinct characteristics regarding their IgE responses, and such characteristics do not necessarily reproduce responsiveness in man qualitatively (is allergen A more potent than allergen B?), let alone quantitatively (allergen A is ten times more potent than allergen B). In fact, strain differences in response to specific allergens have been demonstrated conclusively for certain respiratory allergens [58], and there is no reason to suppose that such differences would not be observed with food allergens. The biology of the IgE response, previously mentioned, also makes it difficult to draw conclusions as to the likelihood that a particular protein will induce an IgE response in man. An important limitation, even if a valid dose–response relationship can be elucidated, is to know what the effective dose is in an individual. This could be affected by factors such as gastric pH and other food components consumed at the same time. Given these factors, an amount which would not induce an IgE response under some circumstances, might do so under a different set of circumstances.

11.4.4.1 *Difficulties in risk assessment.* Allergy risk assessment is a rather new discipline and some of the tools required are either incomplete or absent, because of lack of scientific knowledge. The difficulties pertain to all stages of the risk assessment process, although they increase with progress through the process.

A variety of techniques exist to identify the inherent allergenic potential of a protein, namely comparison of amino acid sequences with known allergens, serum screening, pepsin resistance and animal models. All have inherent drawbacks, which have already been discussed. The current situation is therefore that a judgment is formed regarding the inherent allergenicity of a protein using a weight of evidence approach and the evidence arising from techniques, the predictive ability of which has not been determined. Similarly, hazard characterisation, i.e. estimation of allergenic potency, is subject to limitations. Animals models may provide the current best solution, but the applicability of the findings obtained in inbred animals on a response which depends so heavily on genetic background to man, must always be critically assessed.

Of course, while these approaches give an indication of inherent allergenic potential, they do not take into account the route of exposure. Identification of the hazard is normally the first step in the risk assessment procedure. The next step would be to analyse stability (heat, digestion), the dose levels consumed, susceptible populations, etc. in order to be able to translate hazard into actual risk when consuming the protein. The difficulties inherent in this process can best be illustrated through several examples.

A round table conference with 12 clinical allergists sharing existing data on threshold doses concluded that threshold doses (i.e. lowest doses eliciting a reaction in sensitised individuals) for commonly allergenic foods are finite, measurable and above zero [15]. This was the first time that a group of clinical allergists agreed that threshold doses for allergens even existed. However, agreement was not possible on actual values for the threshold doses for peanut, egg and cow's milk, the foods for which the greatest amount of clinical data were available. The presented data were on elicitation of allergic reactions after challenge. Data on the food allergen doses that may sensitise a subject are very rare, and generally can only be obtained indirectly. For instance, Hill [59] considered possible sensitisation through breast milk, and suggested that very low doses of milk and egg could sensitise. However, how these doses relate to the amounts that may sensitise through ingestion after weaning is unknown. A large area for investigation in this context is the amount of allergen that is bioavailable, as far as the immune system is concerned.

The above illustrates the difficulties of translating hazard into risk. Both the WHO/FAO consultation and the Codex ad hoc working group have suggested that if a protein introduced into a GM-food is a possible allergen, hazard identification is the last step in the risk assessment procedure and the only possible risk management procedure is to discontinue development of the product.

Not all Novel Foods are genetically modified. Over a period of 31/2 years, the EU had 31 applications for approval of Novel Food and Novel Food processes. Of these, 11 were GM foods, 3 new plants, and 17 included new ingredients and processes. Whereas, the possible allergenicity of GM-foods has been extensively discussed, and has resulted in a stringent risk assessment procedure, there has not been a comparable discussion of how to judge possible allergenicity of non-GM novel foods.

The Micronesian nangai/ngali nut (*Canarium indicum*) was not approved as a novel food in the EU on the basis that the allergenicity of ngali nuts had not been investigated and that adequate toxicological data were not available. In a later study,

2/10 grass, birch and mugwort pollen-allergic patients reacted to nangai nuts upon challenge [60]. Several patients in this group probably have allergic reactions to other tree nuts. An unanswered question arising from these new data is whether cross-reactions in pollen-allergic patients are an unacceptable risk, or whether a product like nangai nuts that probably will have a limited consumption, should be judged differently from products such as GM-corn and GM-soy, which may be consumed by the entire population, including infants who are a more susceptible group?

The debate on how to assess and manage the risk of potential allergens in GM-food, other novel food and traditional food is far from over. Clearly, it would benefit from improved understanding of the biology and mechanisms of food allergy.

## 11.5 How can we know whether we have got it right: post-launch monitoring?

Given the limitations of current methods for assessing allergenicity, it is valuable to consider how we could verify that the judgement made was correct. Such verification can provide several advantages. Firstly, it can furnish data about the predictive ability of the methods used. Secondly, it can provide an early indication of potential problems, and permit action to be taken before a widespread public health issue arises. Thirdly, the data generated can, if appropriate, offer the food manufacturer or supplier a sound defence for the use of a novel protein, should its safety be questioned. Allergenicity is an ideal adverse effect to study using such a Post-Launch Monitoring programme: the effects occur very soon after ingestion and can be readily and, ultimately, unequivocally linked to a specific product.

### 11.5.1 *The role of post-launch monitoring*

Post-launch monitoring (PLM) does not substitute for the toxicological safety testing programme but complements it. It recognises that there are two important uncertainties where novel foods are concerned. The first is that the risk assessment presented to support the food contains a number of assumptions about exposure of the population, namely who will consume the food, how often and in what quantities. These assumptions underlie predictions about the extent of known adverse effects. The second uncertainty is, while the aim of toxicological and clinical test programmes is to ascertain the absence of potential adverse effects in representative populations, the diversity of human populations can never be fully reproduced. PLM permits the verification of the assumptions made about population exposure, and hence the validity of the risk assessment. It also permits the detection of unintended effects, such as allergenicity which could not have been identified during the toxicological testing programme or the limited clinical trials in man that precede marketing of a product.

PLM is a term which has been chosen to distinguish it from post-marketing surveillance, which has been a feature of pharmaceuticals for a considerable time. There are a number of distinctions between PLM and post-marketing surveillance, which reflect in particular the different ways in which foods and pharmaceuticals reach those who consume them (Table 11.1).

**Table 11.1** Comparison of post-launch monitoring for foods and post marketing surveillance of pharmaceuticals

| Post marketing surveillance | Post-launch monitoring |
|---|---|
| • Applicable to medicines<br>  • Prescription required<br>  • Use limited (pharmacy)<br>  • Medical condition of user known<br>• Main information channels for feedback: physicians and pharmacists<br>• Information filtered by reporting physicians<br>• Small patient base | • Applicable to functional foods<br>  • No prescription<br>  • Widespread use; no controls on availability<br>  • Medical condition of user unknown<br>  • Main source of information: consumer help lines<br>• Information unfiltered<br>• Large consumer base |

### 11.5.2 *Models of post-launch monitoring*

PLM requires the use, and where necessary the creation, of a variety of communication channels, so that the required information is gathered, processed and then communicated to relevant interested parties. These channels are typically between the company and:

- Consumers (e.g. Company Carelines, household panels, market research companies, data from supermarkets)
- Associations of consumers, in particular special groups of consumers who may be at special risk of certain side effects
- Professional intermediaries (e.g. physicians, nutritionists)
- Regulatory authorities.

Each of these channels produces information of a different nature and degree of quality. When in place, these channels provide a network for detecting potential adverse effects and disseminating that knowledge to protect consumers. An important aspect of PLM is that information from all sources is integrated to provide an overall view of potential problems.

Although Unilever has not commercialised any foods of GM origin, it has gained experience in the PLM of novel foods through the marketing of margarines containing phytosterol esters. The PLM system is built on extensive experience of monitoring the introduction of new products onto the market. It contains three components:

1. Is use as predicted/recommended? Although the data gathered in relation to this question are also used in relation to the commercial aspects of the product, they help to confirm whether the assumptions about exposure used for the risk assessment are valid. Information from this part of the programme is generated primarily through the use of household panels. For instance, in the UK, extensive data are obtained from a superpanel consisting of 10 000 households. This panel, representative of the UK population, generates a detailed demographic and socio-economic picture of the consumers of phytosterol margarines. One limitation of household panels is that they yield data about households, rather than individual consumers. Further qualitative data are therefore obtained by interviewing members of selected households within the panel.

2. Are the known effects and side effects as predicted, both qualitatively and quantitatively? Data obtained in relation to this question will confirm the extent to which the individuals who participated in the clinical trials are representative of the wider population.

3. Does the product induce unexpected side effects? This component is a novel feature of the PLM system and is described below.

The Unilever Careline was selected as the primary communication channel, as prior experience showed that it provides the most extensive coverage of consumers and the most rapid feedback to the company. To date, more than 500 000 calls have been made worldwide to the Carelines for phytosterol margarines. Fewer than 200 calls have been about possible adverse health effects. Other channels included household panels (as above) and random interviewing. Specific studies, monitoring individuals with their explicit knowledge, were found not to be appropriate, as it was found early that the fact of being observed resulted in changes in the observed behaviour (people behaved as the investigators expected them to behave). The product was also discussed with patient associations and specialist and generalist physicians.

The main features of the PLM system are:

1. Specialised training of Careline staff to distinguish calls related to possible health effects from other types. In particular, one aspect of the training covered the posing of questions needed to elucidate from callers as clear a picture of the reported adverse event as possible.

2. Information collected on adverse events is sent quickly to the Unilever Safety and Environmental Assurance Centre (SEAC), which co-ordinates Unilever's safety activities worldwide. There, each event is examined independently by a toxicologist and a clinician.

3. The incidents are then classified as follows:
   - Irrelevant – incident not actually an adverse health effect
   - Not enough information – Careline personnel asked to obtain more information from caller
   - Causal relationship between product use and symptoms can be excluded
   - Possible causal relationship between product use and symptoms
   - Definite causal relationship between product use and symptoms, explained by known properties of ingredient.

4. Investigation of adverse event by physician, if appropriate.

5. Compilation and analysis of data by SEAC to identify possible trends on a worldwide, regional and company basis.

The results of PLM are summarised at three- to six-month intervals for company purposes. These summaries can be made available to Competent Authorities, if this is required as a condition of product approval.

## 11.6 Conclusions

Food allergy is an atopic disease, which although more limited in prevalence than respiratory atopic disease, makes a significant socio-economic impact. Plant-derived allergens make a significant contribution in terms of numbers of allergens, as well as in terms of the number and severity of reactions they provoke. The impact of food

allergy on society has been recognised by all major regulatory authorities, as shown by regulations which require an assessment of the allergenicity of foods classed as novel. No single test or combination of tests can unequivocally predict the allergenicity of a protein which will be ingested. Founded on this recognition, different strategies have been proposed to determine allergenicity. Although these vary in detail between authorities, they reflect a broadly similar approach. The tests in current use have two main objectives:

1. Ascertaining that the novel protein does not provoke reactions in other allergic individuals because of its similarity to known allergens.

2. Establishing the probability that the protein could prove an (novel) allergen. The current consensus recognises that many of the tests in use were developed for other purposes, and require further development and evaluation.

Compared to risk assessment and management of chemicals in food, handling the risk arising from new allergens in food constitutes a very special challenge. This arises for several reasons:

1. Only a part of the population is at risk of being sensitised, and only the sensitised part of the population is at risk of developing disease.

2. Risk identification and characterisation are subject to more uncertainty than for most other areas of toxicology. In contrast to other areas of toxicology, persistent low-dose exposure may exacerbate the problem rather than establish a steady state in which the organism can deal with the toxic insult.

3. In general, the symptoms are acute and caused by one ingestion. The dose causing disease can differ by several orders of magnitude between affected individuals, and may vary within the same individual over time. The symptoms may vary from very mild, e.g. itching of the mouth to death, and there is no way of predicting unequivocally what will occur when a susceptible individual is exposed.

4. Knowledge of the lowest or no observable adverse effect levels (thresholds) in man is very limited, while there is almost no information on sensitising dose(s). In this context, a PLM programme can be a valuable aid to confirm the risk assessment and thereby improve knowledge of the predictive ability of the current testing strategies.

More knowledge on allergens, sensitisation, thresholds, etc. is needed to refine the risk-assessment procedures in food allergy, as well as a more political discussion on the magnitude of risk the society is willing to accept.

## References

1. Jarrett, E.E., Haig, D.M., McDougall, W. & McNulty, E. (1976) Rat IgE production. II. Primary and booster reaginic antibody responses following intradermal or oral immunization. *Immunol* **30(5)**, 671–677.
2. Heppell, L.M., Cant, A.J. & Kilshaw, P.J. (1984) Reduction in the antigenicity of whey proteins by heat treatment: a possible strategy for producing a hypoallergenic infant milk formula. *Br J Nutr* **51(1)**, 29–36.

3. Poulsen, O.M. & Hau, J. (1987) Murine passive cutaneous anaphylaxis test (PCA) for the 'all or none' determination of allergenicity of bovine whey proteins and peptides. *Clin Allergy* **17(1)**, 75–83.
4. Fritsché, R. & Borel, Y. (1994) Prevention of allergic sensitization to beta-lactoglobulin with conjugates made of beta-lactoglobulin coupled to isologous immunoglobulin G. *J Allergy Clin Immunol* **93(4)**, 778–786.
5. Lewis, S., Butland, B., Strachan, D. *et al*. (1996) Study of the aetiology of wheezing illness at age 16 in two national British birth cohorts. *Thorax* **51**, 670–676.
6. Nordlee, J.A., Taylor, S.L., Townsend, J.A., Thomas, L.A. & Bush, R.K. (1996) Identification of a Brazil-nut allergen in transgenic soybeans. *N Engl J Med* **334(11)**, 688–692.
7. Melo, V.M.M., Xavier-Filho, J., Lima, M.S. & Prouvost-Dannon, A. (1994) Allergenicity and tolerance to proteins from Brazil nut (*Bertholletia excelsa* H.B.K.). *Food Agric Immunol* **6**, 185–195.
8. Metcalfe, D.D., Astwood, J.D., Townsend, R. & Sampson, H.A. (1996) Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Rev Food Scien Nutr* **36(S)**, 165–186
9. Astwood, J.D., Leach, J.N. & Fuchs, R.L. (1996) Stability of food allergens to digestion *in vitro*. *Nat Biotechnol* **14(10)**, 1269–1273.
10. Evaluation of Allergenicity of Genetically Modified Foods, FAO/WHO Expert Consultation on Foods Derived from Biotechnology, Rome 22–25 January 2001. FAO, Rome, Italy.
11. Aalberse, R.C. (2000) Structural biology of allergens. *J Allergy Clin Immunol* **106(2)**, 228–238.
12. Strobel, S. & Mowat, A.M. (1998) Immune responses to dietary antigens: oral tolerance. *Immunol Today* **19(4)**, 173–181.
13. Arps, V., Sudowe, S. & Kolsch, E. (1998) Antigen dose-dependent differences in IgE antibody production are not due to polarization towards Th1 and Th2 cell subsets. *Eur J Immunol* **28(2)**, 681–686.
14. Rogers, P.R. & Croft, M. (1999) Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J Immunol* **163(3)**, 1205–1213.
15. Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C. *et al*. (2002) Factors affecting the determination of threshold doses for allergenic foods: How much is too much? Report of an international conference. *J Allergy Clin Immunol* **109(1)**, 24–30.
16. Hourihane, J.O., Kilburn, S.A., Nordlee, J.A., Hefle, S.L., Taylor, S.L. & Warner, J.O. (1997a) An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomised, double-blind, placebo-controlled food challenge study. *J Allergy Clin Immunol* **100**, 596–600.
17. Oppenheimer, J.J., Nelson, H.S., Bock, S.A., Christensen, F. & Leung, D.Y.M. (1992) Treatment of peanut allergy with rush immunotherapy. *J Allergy Clin Immunol* **90**, 256–262.
18. Bjorksten, B. (1994) Genetic and environmental interaction in children. In: *Progress in Allergy and Clinical immunology*, Vol. 3, Johansson, S.G.O. (ed.). Hogrefe and Huber Publishers, pp. 97–103.
19. Bernhisel-Broadbent, J. (2001) Diagnosis and management of food allergy. *Curr Allergy Rep* **1(1)**, 67–75.
20. Young, E., Stoneham, M.D., Petruckevitch, A., Barton, J. & Rona R. (1994) A population study of food intolerance. *Lancet* **343**, 1127.
21. European Commission (1997a) Regulation (EC) No 258/97 of the European Parliament and of the Council concerning novel foods and novel food ingredients. *Official Journal of the European Communities* **L43**, 1–7.
22. Estruch, J.J., Carozzi, N.B., Desai, N., Duck, N.B., Warren, G.W. & Koziel, M.G. (1997) Transgenic plants: an emerging approach to pest control. *Nat Biotechnol* **15(2)**, 137–141.
23. Atkinson, H.A. & Miller, K. (1994) Assessment of the brown Norway rat as a suitable model for the investigation of food allergy. *Toxicology* 12; **91(3)**, 281–288.
24. Commission recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No. 258/97 of the European Parliament and of the Council. *Official J* **L253**, 16 September 1997, 1–36.
25. Vieths, S., Aulepp, H., Becker, W-M. & Buschmann, L. (1996) Characterization of labile and stable allergens in foods of plant origin. In: *DFG symposium: Food Allergies and Intolerances*. Bonn 8–10 May 1995. VCH Publishers, Weinheim, New York, pp. 130–149.
26. Leung, P.S., Chow, W.K., Duffey, S., Kwan, H.S., Gershwin, M.E. & Chu, K.H. (1996) IgE reactivity against cross-reactive allergen in crustacea and mollusca: evidence for tropomyosin as the common allergen. *J Allergy Clin Immunol* **98** (5 Pt 1), 483–487.
27. Gendel, S.M. (1998) Sequence databases for assessing the potential allergenicity of proteins used in transgenic food. *Adv Food Nutr Res* **42**, 63–92.

28. Codex ad hoc Intergovernemental Task Force on Foods Derived from Biotechnology, *Ad Hoc* Open-ended Working Group on Allergenicity, Report of the working group, September 12, 2001.
29. Ouellette, B.F.F. (1998) The GenBank sequence database. In: *Bioinformatics. A Practical Guide to the Analysis of Genes and Proteins*. Baxevanis, A.D. & Ouellette, B.F.F. (eds). John Wiley and Sons Inc., New York.
30. Pearson, W.R. & Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Science* **227**, 1435–1441.
31. Karlin, S. & Altschul, S.F. (1990) Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. *Proc Natl Acad Sci USA* **67**, 2264–2268.
32. Elsayed, S., Apold, J., Holen, E., Vik, H., Floorvaag, E. & Dybendal, T. (1991). The structural requirements of epitopes with IgE binding capacity demonstrated by three major allergen from fish, egg and tree pollen. *Scand J Lab Invest* **51** (Suppl 204), 17–31.
33. Baderschneider, B., Crevel, R.W.R., Earl, L.K., Lalljie, A., Sanders, D.J., & Sanders, I.J. (2002) Sequence analysis and resistance to pepsin hydrolysis as part of an assessment of the potential allergenicity of Ice Structuring Protein Type III HPLC 12. *Food Chem Toxicol* **40**, 965–978.
34. Barker, W.C., Garavelli, J.S., Hou, Z. *et al*. (2001) Protein Information Resource: A community resource for expert annotation of protein data. *Nucleic Acids Res* **29**, 29–32.
35. Burks, A.W., Shin, D., Cockrell, G., Stanley, J.S., Helm, R.M. & Bannon, G.A. (1997) Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* **245**, 334–339.
36. Stanley, J.S., King, N., Burks, A.W. *et al*. (1997) Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. *Arch Biochem Biophys* **342**, 244–253.
37. Bindslev-Jensen, C.B., Sten, E., Earl, L.K. *et al*. (2003) Assessment of the potential allergenicity of Ice Structuring protein Type III using the FAO/WHO 2001 decision tree for novel foods. *Food Chem Toxicol* **41**, 81–87.
38. Becker, W.-M. (2001) Sequence homology and allergen structure. Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, www.who.int/fsf/Gmfood/Documents_list.htm.
39. Poulsen, L.K., Pedersen, M.F., Malling, H.-J., Søndergaard, I., & Weeke, B. (1989) Maxisorp RAST. A sensitive method for detection of absolute quantities of antigen-specific IgE. *Allergy* **44**, 178–189.
40. Koppelman, S.J., Knulst, A.C., Koers, W.J. *et al*. (1999) Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *J Immunol Methods* **229(1–2)**, 107–120.
41. Ebbesen, F. & Skov, P.S. (1986) Basophil histamine release in cord blood. *Allergy* **41**, 562–567
42. Hoffmann, A., Vieths, S. & Haustein, D. (1997) Biologic allergen assay for *in vivo* test allergens with an *in vitro* model of the murine type I reaction. *J Allergy Clin Immunol* **99(2)**, 227–232.
43. United States Pharmacopoeia, Vol. XXII, NF XVII, Rockville, MD, 1990, 1788.
44. Wal, J.M. (2001) Structure and function of milk allergens. *Allergy* **56**(Suppl 67) 35–38.
45. Dearman, R.J., Caddick, H., Stone, S., Basketter, D.A. & Kimber, I. (2001) Characterisation of antibody responses induced in rodents by exposure to food proteins: influence of route of exposure. *Toxicology* **167**, 217–231.
46. Scientific advisory panel report No. 2000–06, Assessment of Scientific Information Concerning StarLink™ Corn. US Environmental Protection Agency 2000.
47. Minekus, M., Marteau, P., Havenaar, R. & Huis in't Veld, J.H.J. (1995) A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. *ATLA* **23**, 197–209.
48. Miller, K., Meredith, C., Selo, I. & Wal, J.-M. (1999) Allergy to bovine ß-lactoglobulin: specificity of immunoglobulin E generated in the Brown Norway rat to tryptic and synthetic peptides. *Clin Exp Allergy* **29**, 1696–1704.
49. Knippels, L.M.J. (1998) Oral sensitisation to food proteins and immune mediated effects; a Brown Norway rat food allergy model. Thesis, University of Utrecht, ISBN 90–39–31–5760.
50. Li, X.-M., Serebrisky, D., Lee, S.Y. *et al*. (2000) A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergens mimic human responses. *J Allergy Clin Immunol* **106**, 150–158.
51. Knippels, L.M.J., Penninks, A.H., Spanhaak, S. & Houben, G.F. (1998) Oral sensitization to food proteins: a Brown Norway rat model. *Clin Exp Allergy* **28**, 368–375.
52. Li, X.-M., Schofield, B.H., Huang, C.-K., Kleiner, G.I. & Sampson, H.A. (1999) A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol* **103**, 206–214.
53. van Halteren, A.G.S., van der Cammen, M.J.F., Biewenga, J., Savelkoul, H.F.J. & Kraal, G. (1997) IgE and mast cell responses on intestinal allergen exposure: A murine model to study the onset of food allergy. *J Allergy Clin Immunol* **99**, 94–99.
54. Atkinson, H.A.C. & Meredith, C. (1998) Assessment of allergenic potential of novel proteins in food crops using the brown norway rat model. *Proc 3rd Karlsruhe Nutrition Symposium*, October, 70–75.

55. Atkinson, H.A.C., Johnson, I.T., Gee, J.M., Grigoriadou, F. & Miller, K. (1996) Brown Norway rat model of food allergy: effect of plant components on the development of oral sensitization. *Food Chem Toxicol* **34(1)**, 27–32.
56. Meredith, C. & Atkinson, H. (2000) Development of methods to predict the allergenic potential of genetically modified foods and novel protein products. *Toxicology* **148**, 41–42.
57. Zhang, J.-G. & Crevel, R.W.R. (1999) The IgG response of Brown Norway rats to oral administration of ovalbumin. Human and Exp. *Toxicology* **18**, 528.
58. Blaikie, L. & Basketter, D.A. (1999) Strain variation in the IgG1antibody response to proteins administered intranasally in the mouse. *Food Chem Toxicol* **37**, 897–904.
59. Hill, D.J. (2001) Prevalence of allergen in Food and Threshold for Sensitization. Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, www.who.int/fsf/Gmfood/Documents_list.htm.
60. Sten, E. (2001) Cross-reactive epitopes in the Micronesian nut nangai (*Canarium indicum*) can cause clinical allergic reactions. 8th International Symposium on immunological, chemical and clinical problems of food allergy, Venezia, 11–13.3 (poster).

# 12 Monitoring of and Technological Effects on Allergenicity of Proteins in the Food Industry

HARRY WICHERS, ARIETTE MATSER, AART VAN AMERONGEN, JAN WICHERS and CRISTINA SOLER-RIVAS

## 12.1 Introduction

The prevalence of allergies seems to be increasing in industrialised countries [1]. This applies in particular for respiratory tract allergies. However, the incidence of food-related allergies also appears to be increasing [2], although sometimes reports may seem somewhat conflicting [3]. It is not always clear, for instance, whether improved detection methods, or increased awareness of the phenomenon, plays a quantitative role at the epidemiological level. Nevertheless, it is undeniable that food-related allergies present, to say the least, considerable discomfort to sensitive individuals.

Food processing to develop hypoallergenic foods is a relatively novel and not very widely used approach to decrease the incidence of food allergy symptoms. Only a few examples exist of hypoallergenic foods that were brought to the market, proteolytically hydrolysed milk proteins for infant food formulations perhaps being the best known. Solutions for clinically diagnosed individuals are usually sought by protection, i.e. contact between allergenic food and sensitive individual, and adequate labelling of food products. On the one hand, the relatively small number of hypoallergenic food products in the market may be the reflection of the relatively small number of individuals who are sensitive to a specific food product. On the other hand, allergens are often considered to be resistant to processing [4, and references therein]. Although this assumption may at first seem reasonable, other options for specific processing and new perspectives to reduce allergenicity of foods have recently been discussed [5, 6].

This review deals with the prospects and promises that the combination of the identification and monitoring of allergens (translated into immunochemical assays), and classical and novel food processing technologies may offer to produce food products with reduced allergenicity. Finally, prospects for the integration of the rapidly emerging genomics and bioinformatics research areas with food technology to reduce allergenicity of foods are discussed.

## 12.2 Structural basis of allergenicity

### 12.2.1 *Allergenic proteins and epitopes*

Allergic reactions to foods are in fact allergic reactions to individual food components, in most cases proteins [7], but sometimes also carbohydrates [8]. Allergens are often proteins with molecular masses of 10–70 kDa [7, 9–11], but they may occur as multimers such as the peanut allergen *Ara* h 1 [7, 11] (see Section 12.4.2).

On such allergens, epitopes can be found that provoke the IgE-mediated allergic response [4, 7, 9–11]. Such T-cell epitopes are generally small, 12 to 18 residue, peptide fragments [7, 11]. Food allergens and their epitopes may be quite resistant and survive the effects of food processing and digestion [4, 12].

The integrity of some epitopes is not exclusively determined by their primary structure. So-called conformational epitopes are also characterised by the tertiary structure of a protein, and their integrity therefore depends on the thermodynamics that determines tertiary protein structure [7].

Despite the relative stability of allergens and epitopes to digestion and processing, knowledge of the epitope structure and the factors that determine its chemical and physical stability can lead to the design of specific and well-directed approaches to decrease food allergenicity. Structural similarities between allergens and their epitopes may lead to the development of more generic approaches to alter their allergenicity.

### 12.2.2 *Allergenic cross-reactivity, biological function and processing*

Cross-reactivity between allergens and epitopes from various food products may occur, as described for example for allergens from crustaceans [13] and from legumes [12, 14]. Also, cross-reactivities for respiratory allergens and food allergens have been well described, for instance between allergens from pollen of birch (Bet v 1) and mugwort (Art v 1), and allergens from apple (Mal d 1), carrot (Dau c 1), potato [15, 16], pear (Pyr c 1) [18], mango, celery (Api g 1) [8, 19] and hazelnut [20].

Allergenic proteins can be grouped into a limited number of families, based on their allergenic cross-reactivities (Fig. 12.1) [21]. Their common properties coincide to a considerable extent with the biological functions of these proteins *in planta*, for instance as pathogenesis-related (PR) and defence proteins (Bet v 1 family (PR10), Bet v 2 family (profilins) and LTPs (PR14)), or storage proteins (the 7S/11S globulins and 2S albumins).

The grouping of allergens on the basis of their resistance to heat treatment, however, shows a less consistent picture, at least for the Bet v 1 family. The dominant allergen of apple, Mal d 1, which is immunologically cross-reactive to Bet v 1 [15, 16], is rather easily destroyed upon heating [20]. This also applies to the Bet v 1 equivalent from celery, Api g 1 [8], but not to the major allergens from carrot (Dau c 1), which was shown to be heat stable for 40 minutes at 100°C [20] or even when autoclaved at 121°C [20, 22]. Hazelnut allergens of Mr 14–18 kDa were heat stable for 15 minutes at 155°C [20].

These observations lead to the conclusion that proteins with similar biological functions, biochemical characteristics such as molecular weight (e.g. the Bet v 1 family is composed of proteins of ca. 14–18 kDa) and immunological cross-reactivity, nevertheless may vary considerably in their physicochemical stability.

## 12.3 Analytical approaches to identify and monitor allergenicity

In recent years, the development and commercialisation of rapid and relatively cheap immunochemical assays for a variety of applications has been accomplished. Such

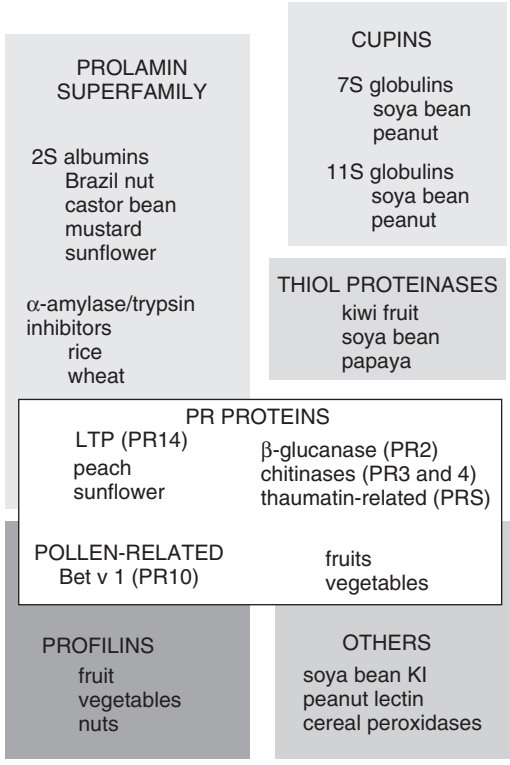


**Fig. 12.1** Classification of plant proteins on the basis of their allergenic cross-reactivity and biological function. Based on: Mills *et al.* [21].

assays may also hold promise for use in monitoring the fabrication of foods with reduced allergenicity, in particular if (pooled) patient's sera could be used. However, it should always be remembered that immunochemical assays remain *in vitro* assays. A method of testing that is more closely linked to the *in vivo* situation, such as skin prick testing, will be required to validate the predictive power of the *in vitro* tests. In particular in the case of foods which, on the basis of *in vitro* testing, are considered to have reduced allergenicity, it literally applies that 'the proof of the pudding will be in the eating': double-blind placebo-controlled food challenge testing will remain an essential element in the preparation of safer foods for sensitive individuals.

### 12.3.1 *Immunochemical assay technology*

An immunoassay is defined as an analytical technique which uses antibodies and/or antibody-related reagents for the selective determination of sample components [23]. A key feature which distinguishes immunoassay kits from other modern techniques of analysis is that in an immunoassay the high technology is built into the molecules and not into the apparatus [24]. The basis of specificity and sensitivity is the antibody–antigen reaction, which offers the possibility to detect components at levels as low as

$10^{-12}$ M. Immunoassays are relatively rapid and can have a high throughput. Although the initial cost of immunoassay development may be rather high, the cost per test is often a fraction of that for other analytical methods, when the procedure is well established [25].

### 12.3.2 *Stages of development*

Critical phases in the development of immunoassay systems are: (1) the preparation and purification of the immunogen/antigen, (2) the production of antibodies, (3) antibody screening and (4) assay development [25]. Two additional phases have been defined for the commercial development of immunoassay kits: (5) assay validation and standardisation, and (6) commercial manufacture [26]. Some of these aspects will be addressed in more detail below. Evaluation of an immunoassay kit by endusers is essential to define the usefulness in a particular situation and under specific conditions. Criteria for evaluation are [27]:

Usefulness:

- Cost (in terms of reagents, equipment and facilities, speed of analysis)
- Use (in the laboratory or field, by trained or untrained analyst)
- Stability (shelf life)
- Quality assurance/Quality control (requirements, protocols, cost, availability of standards).

Confidence factors:

- Bias, precision, accuracy, specificity
- Limit of detection/determination
- Repeatability/reproducibility (i.e. within or between laboratory coefficients of variation)
- False positive/negative results.

An overall rating must be based on the relative importance of the criteria that will vary in relation to the specific use and circumstances.

12.3.2.1 *Important parameters in choice of assay format.* Several parameters have to be evaluated in the choice of a particular assay format. These parameters include:

- Consistency of the product
- The availability of the target compound in the product at particular stages of processing
- The type of target compound (a protein in the case of allergens)
- Concentration of the target compound in relation to assay sensitivity
- Type of data required (quantitative or semi-quantitative)
- Skills of people available for testing
- Field or laboratory conditions
- Required speed of data availability and
- Acceptability of costs of screening with respect to product value.

A balanced evaluation of these parameters will successfully guide the selection of an appropriate assay format that meets the requirements of the users.

12.3.2.2 *Immunoassay formats.* Over the last 15 years many immunoassays have been developed to target components in foods [28, 29]. Most of these assays are of the ELISA type, although the number of other formats is rapidly growing. Two related formats used in allergy screening are the Radio-Allergo-Sorbent Test (RAST) and the Enzyme-Allergo-Sorbent Test (EAST). Antibodies are also used in affinity chromatography for the specific purification of target compounds. Furthermore, immunoblotting technology offers good possibilities for screening sera for immunoreactivity against a large number of proteins (allergens).

In addition to these methods, more sensitive assays such as those with fluorescent and chemiluminescent signal detection have appeared on the market. The superior sensitivity of these methods allows the determination of very dilute components, which is a major advantage particularly in the case of toxic materials. Furthermore, assays incorporating new detection principles have taken over part of the market in food analysis. These new immunosensor assays use, for example, optical, electrochemical and piezoelectric detection principles [30] combined with flow-injection analysis. These assays offer shorter assay times and the possibility to analyse the real-time kinetics of the antibody–antigen interaction. Another recent development in food analysis is the introduction of rapid and simple assays in which *dry-chemistry* forms the basis of a homogeneous one-step assay. In this respect, formats like the dipstick assay and the lateral flow-through device are being used. An important advantage of these assays is the fact that untrained people can perform the test and interpret the results. The over-the-counter (OTC) pregnancy hormone test kit is the classical example of this kind of immunoassay [31]. An additional benefit is the possibility to develop multi-analyte one-step assays monitoring several components in a single sample [32].

### 12.3.3 *Crucial aspects in the development of immunoassays for food allergens*

In the development of an immunoassay for a food allergen, several factors need to be addressed that are crucial for a successful market introduction. A number of these will be briefly discussed.

12.3.3.1 *Type of immunoassay.* The type of immunoassay format is important with respect to the way an allergen is presented to specific antibodies. Factors inherent to the various formats may influence the binding of the antibodies, such as (partial) denaturing of proteins upon immunoblotting and the effect of *dry-chemistry* conditions in one-step lateral flow immunoassays. If an allergen is sensitive to denaturation, this phenomenon should be taken into account.

12.3.3.2 *Coating antigen.* A coating antigen may be used to generate a 100% reference signal, especially in competitive immunoassay formats. The interaction of the antibodies used in the assay with this coating antigen and the interaction with the allergen in the sample food matrix should at least be similar in order to develop an assay with reasonable sensitivity. Several preparations may be used as coating antigen: an extract of the food matrix containing the allergen, a purified allergen, an allergenic hydrolysate, or synthetic peptides based on the allergen, or a mimotope peptide that interacts favourably with the antibodies (possibly generated via phage display peptide libraries). In this

respect, it should be noted that the immobilisation of the coating allergen and the consequences for its 3D structure and recognition by the antibody need to be studied for optimal results in assay development.

12.3.3.3 *Antibodies.* The type and desired characteristics of the antibodies that are used in a particular assay are crucial for correct interpretation of the immunoassay results [33, 34]. Antibodies induced in animals upon immunisation with the allergen can be a good source of ligands that recognise the allergen in food samples. In particular, the production of a monoclonal antibody guarantees the lasting availability of an important assay reagent.

However, the interaction of these antibody preparations will not necessarily reflect the immunoreactivity of IgE in patients. In particular cases this could lead to undesired or misleading results, for example if an assay is meant to screen for reduction or elimination of allergenicity as a result of processing (see also Section 12.3.4). The assemblage of an antibody source covering all or the most important allergenic determinants based on pooled human sera may be an alternative. Using these antibodies, the assay results will reflect as closely as possible the way a patient would react upon exposure to the allergen. This antibody source, however, is limited and new batches have to be prepared on a regular basis. In addition, it will be very difficult to prepare a fully representative sample of IgE reactivities of patients [35].

12.3.3.4 *Sensitivity and data output.* The required sensitivity will also determine the possible immunoassay formats [31]. If a very sensitive assay is desired, the development of a rapid one-step lateral flow assay may not be relevant. Furthermore, a one-step assay is also not suitable if quantitative data output is essential, since it gives a yes or no answer. Therefore, it is necessary to formulate sensitivity levels for each allergen and to indicate whether signals should be quantitative or only semi-quantitative. For a given allergen this information may differ between various products. Furthermore, if the level of an allergen is too low to be measured by the diagnostic assay, an indicator compound should be identified whose concentration shows a good correlation with that of the allergen.

12.3.3.5 *Sample preparation.* In general, sample preparation for immunoassay detection is quite simple compared to other analytical techniques. However, if the sample of interest is a food material or product, this vital step in the performance of an immunoassay is not always straightforward. Extraction of the target compound may involve enzymatic breakdown of cell walls or other compartments, or include a step with an organic solvent. This may affect the integrity of the allergen and, consequently, the recognition by the antibodies in the immunoassay. In this respect, the interaction of an allergen with other compounds in the food matrix needs to be studied as well. Such interacting compounds may mask the allergenic determinants resulting in reduced or impaired binding with the antibodies.

### 12.3.4 *Trends in allergy research and implications on immunoassay development*

12.3.4.1 *Processing to reduce allergenic potential.* Over the last years, there has been a growing interest in the possibility of reducing or eliminating the allergenic

potency of proteins by means of processing. This would make it possible to use such processed food materials in many more products without a risk of adverse reactions of allergic consumers. However, an appropriate assay is required to monitor the reduction or elimination of allergenicity. In these cases detection of the allergen or another component from the food product containing the allergen is not sufficient. Detailed information should be available on the common (and rare) allergenic determinants, as shown by the immunoreactivities of patients towards the allergen. It implies that the antibodies used in such assays make it possible to monitor residual allergenic potency of the processed allergen (see also Section 12.3.3.3). The reliability of the diagnostic assay should also be high, especially in cases where low amounts of allergens may result in severe effects in patients. To allow the introduction of immunoassays to monitor allergenic potential (after processing), future research should be directed to further characterise the allergenic determinants of a range of allergens that could possibly be targets for elimination by processing. In this respect the food industry should adopt protocols to track and trace food allergens, in order to guarantee correct and efficient monitoring.

However, it should be questioned whether processing as a means of reducing allergenicity should be applied to food products containing allergens that may elicit severe responses in patients. Because it is doubtful that processing will ever result in 100% reduction of allergenic potential, the particular allergen, and perhaps even its source, should be completely absent from the food product in these cases. Consequently, a relevant immunoassay should be able to monitor the presence of the allergen.

12.3.4.2 *Home testing with simple and rapid assays.* The market for *over-the-counter* home testing human diagnostics is rapidly increasing. Although not very prominent yet, further growth in simple and rapid diagnostic kits for food or allergen monitoring by the consumer is expected. In view of the complexity of food matrices and the problems that may arise upon extraction of an allergen from these, it may be questioned whether a diagnostic kit should be available for each allergen. A false negative result upon testing a food product may give consumers the impression that they are dealing with a safe product. In particular cases, this may even lead to serious complications and perhaps to legal action against the diagnostic company that produced the test. Therefore, the characteristics of OTC test kits for the monitoring of food allergens should be carefully evaluated in order to determine whether commercial availability is justified and advisable for each *allergen-product* combination.

## 12.4 Specific approaches to reduce allergenicity

Food processing can aim to reduce allergenicity by irreversible removal of allergenic proteins or by modifying the allergen structure in such a way that the allergenic epitopes are no longer recognised by the immune system. These objectives can, in principal, be achieved by a number of methods:

- Chemical
- Biochemical (proteases, oxidases)

- Physical (heating, novel processing, extraction)
- By breeding: classical, mutation breeding or by genetic modification.

The acceptable rigorousness of processing methodology is limited, however, because of *processing side effects* that cause unacceptable sensory losses (e.g. in colour or taste) or that cause loss of desired processing properties (e.g. swelling behaviour, baking properties of flour, etc.). The method of choice for processing will therefore depend on a thorough evaluation of raw material characteristics, and product specifications: Will the product present a more or less homogeneous phase, such as a juice or a mash, or more or less intact tissue, such as processed fruits? Is thorough physical processing acceptable, or is there a chance to apply more specific biochemical processing, such as enzyme treatment?

Until now, the approach to produce products with a decreased allergenicity has largely been empirical. The main reason for this is a lack of detailed knowledge on integral allergen and epitope structure, which hampers the design of more rational, generic strategies for processing. Examples of the use of processing to decrease allergenicity will be discussed, together with the potential that novel types of processing may offer.

### 12.4.1 *Hypoallergenic apple products*

The cross-reactivity between birch pollen allergy and apple allergy is well described as already discussed to a considerable extent in Section 12.2. The major allergen from apple is the ca. 18 kDa Bet v 1 analogue Mal d 1 [15–17], although a few minor allergenic proteins of Mrs of 30, 37, 43 and 67 kDa have been identified [20].

Apple is an interesting model system for studying processing effects, because it is consumed in a large variety of processed forms (fresh, after storage, as sauce, as juice), with varying physicochemical states (from intact fruit to a physically homogeneous form such as juice).

Freshly cut apple loses a considerable part of its IgE-binding capacity [20, 36]. This seems related to oxidation processes in the apple tissue, that are probably enzymatically catalysed [36]. It can therefore be anticipated that oxidised endogenous phenolic compounds such as catechin can bind to specific amino acid residues in the epitopes, thereby reducing their IgE-binding capacity and, potentially, their allergenicity. The role of specific oxidising enzymes, such as polyphenol oxidases and peroxidases, is currently under investigation.

Mal d 1 is relatively heat labile, and pasteurisation was found to be decisive for elimination of its IgE-binding capacity [20]. This also applies for the Bet v 1 analogue from celery, Api g 1 [8], but contrasts with the heat stability of peach, carrot and hazelnut allergens that are also included in the Bet v 1 family [20, 22].

Enzymatic processing, either with the aid of proteases or specific oxidases, may enable the development of a successful processing method, provided sufficient contact between the allergen and enzyme can be ensured.

### 12.4.2 *Hypoallergenic peanut products*

Peanut allergy, being frequent in the United States but much less so in Europe until the mid-1980s, is increasingly becoming a problem in industrialised countries. Severe

effects, including anaphylactic shock, have been described. Approximately 0.5–0.7% of the general population appears sensitive, and there is an association between asthma and peanut allergy [37].

Ara h 1, a major peanut allergen belonging to the vicilin family of seed proteins (see Section 12.2), has been described relatively well [38, 39]. Using serum IgE from peanut hypersensitive patients, at least 23 different IgE-specific epitopes were mapped throughout the length of the protein. All of the epitopes were 6–10 amino acids in length [38]. Analysis of Ara h 1 purified from raw and heat-treated material showed an irreversible transition between 80 and 90°C (increase in β-structure and aggregation), but this did not affect IgE binding [39]. Apparently, the correct conformation of epitopes is not crucial and IgE is still able to bind to the relatively short, abundant and apparently heat stable linear peptide sequences in the denatured protein [39].

Ara h 1 is a highly stable homotrimer, held together by hydrophobic interactions [40]. The hydrophobic amino acids that stabilise the trimer are at the distal ends of the three-dimensional structure, where monomer–monomer contacts occur. Also the majority of IgE-specific epitopes are located in this region. In this way, the tertiary and quaternary structures of the protein may contribute to its allergenicity, as digestion yields various IgE-specific protease resistant fragments [40].

Nevertheless, some approaches can be followed to produce hypoallergenic peanut products. Thorough extraction of protein from peanut oil results in a product that is harmless to sensitive individuals [41, 42]. Substitution of critical amino acids in epitopes has been shown to influence the binding strength of antibodies [34, 43, 44]. It was also shown that substitution of some critical residues in IgE-specific epitopes led to loss of IgE binding, illustrating the potential use of breeding to produce non-allergenic peanuts [44]. Last, but not least, a differential effect of heating on the IgE-binding capacity of peanut preparations was shown [45]. There was significantly less IgE binding to Ara h 1, Ara h 2 and Ara h 3 in fried and boiled preparations as compared to dry roasted peanuts, although roasting uses higher temperatures. It is possible that the formation of Maillard reaction-related neoallergens plays a role [6]. Dry roasted peanuts are the predominant consumed form in the United States, in contrast to China. Peanut allergy is also much more prevalent in the United States than it is in China. If this apparent correlation can be further substantiated, it would illustrate the potential to reduce food allergic reactions through the application of specific processing methods.

### 12.4.3 *Hypoallergenic soy products*

Soy allergy belongs to the so-called *big eight* of food allergies, as does peanut allergy. A variety of methods, ranging from breeding-oriented to extraction, have been successfully applied to produce low-allergenicity soybean products.

Refined, bleached and deodorised soy oils did not pose a risk to allergic individuals [42]. Heating of soy proteins at 80 or 120°C also significantly reduced IgE binding to allergenic epitopes, to a greater extent than IgG binding which was used as an indicator of the integrity of immunogenic epitopes. Also enzyme digestion was claimed to greatly reduce IgE binding [46]. This latter option was confirmed by other researchers [47, 48].

A very interesting study was presented by Ogawa *et al*. [47]. The major allergens in soy are the storage proteins Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K. The allergenicity of soybean and soybean products was reduced by a combination of mutation breeding, physicochemical treatment and enzymatic digestion. Gly m Bd 60K and Gly m Bd 28K were eliminated by development of a mutant line, while the strongest allergen, Gly m Bd 30K was salted out or destroyed by enzymatic digestion. Challenge tests showed that approximately 80% of soy sensitive patients could ingest products that were produced from this hypoallergenic soy, without adverse reactions [47].

### 12.4.4 *Enzymatic processing*

Biochemical (enzymatic) processing of raw materials may also aid the removal of allergenicity. Prerequisites are sufficient contact between the allergen, or epitope, and the enzyme, and sufficient control of undesired side effects that may affect sensory quality or processing properties.

In some cases, proteolytic processing is insufficient to reduce allergenicity, as described above for peanut [40] and as also described for peach products [22]. Again, insufficient contact or insufficiently specific proteases may account for the lack of success.

Another clear example of the potential use of a specific protease is presented by the decreased allergenicity of wheat flour gluten, via treatment with the protease bromelain [49, 50]. The wheat glutenin IgE-binding epitope has the structure Gln-Gln-Gln-Pro-Pro [50], which made it susceptible to cleavage by bromelain, a peptide that cleaves near Pro-residues. The IgE-ELISA test used in the experiments suggested that the treatment resulted in hypoallergenic flour. A similar result was achieved with the (non-food grade) enzyme collagenase from *Clostridium*. This suggests that epitopes with the structure Gln-X-Y-Pro-Pro may be sensitive to proteolytic modification. An undesirable side effect of this type of proteolytic treatment may be that the proteases will change the structure of the bulk of the available protein, thereby affecting the baking quality of the flour. Although many proteins were indeed degraded, Tanabe *et al*. produced a muffin from the hypoallergenic flour [49].

A further example of successful enzymatic processing is the production of hypoallergenic rice by a two-stage enzymatic process, making use of actinase. The treated rice grains retained acceptable textural properties [51, 52].

The potential, in specific cases, of using oxidising enzymes to reduce allergenicity has already been illustrated for apple (Sections 12.2 and 12.4.1). Hazelnut has also been treated successfully to reduce its allergenicity, via treatment with trypsin, elastase or a protease mixture [20].

### 12.4.5 *The use of breeding and genetic modification to reduce food allergenicity*

The potential for using breeding to reduce the allergenicity of food products has already been illustrated for peanut (epitope amino acid replacement) [44] and soy (chemical mutation induction) [47]. These examples, together with the demonstration that proteolytic modification of an immunogenic epitope can be achieved

*in vitro* without altering enzyme activity [5], illustrate that structural modifications in proteins can be introduced without unacceptably altering the overall protein properties.

Genetic modification also holds promise for quality improvement of foods from an allergenicity perspective. Genetic modification was successfully applied to reduce allergenicity of rice, by knocking out an α-amylase [53]. Less successful, although not aimed at reducing allergenicity but at increasing the sulphur-content of soy, was the introduction of a gene from Brazil nut. A strong allergic response to the introduced protein, which was a known allergen, was indeed observed [7].

An intense debate is currently ongoing as to the allergenic safety of genetically modified (GM) foods. Indications that any risk is controllable and should not be exaggerated are supported by the existence of decision trees that can be used to rationally estimate the potential allergenicity of not only GM foods, but any kind of novel foods [21]. Until now, there are no reports on abnormal health effects due to, for example, the market introduction of GM soy products in the United States. A comparison between natural soybean and glyphosate-resistant GM soybean showed no difference in allergens [54].

## 12.5 Potential of novel processing

As markets become more consumer-driven than ever, novel preservation methods are being developed. The aim is to pasteurise or sterilise food products with less thermally induced quality loss, by using chemical additives to give a better quality compared to conventional preservation methods. The most important new technologies are high pressure processing (HPP) and pulsed electric field treatment (PEF).

Conventional heat treatments result in inactivation of micro-organisms and enzymes. However, these treatments may also cause undesired alteration of the product. Minimal processing techniques have less effect on the quality of the fresh materials by processing the products at room temperature. Hurdle technology is an example of such a mild preservation technique: through the combined application of mild physical processing techniques and, for instance, low doses of natural preservatives, synergistic effects are achieved with less impact on the sensory characteristics of the raw material. The treated product combines high quality (preservation of the fresh characteristics) with microbiological and enzyme inactivation.

### 12.5.1 *High pressure processing*

Pressures up to 1000 MPa can be applied to liquids for food conservation and preparation. Under these high pressures, macromolecules may be altered, with protein denaturation, lipid crystallisation, starch gelatinisation and other effects. Small molecules associated with flavours, colours and vitamins are not affected (Fig. 12.2). Therefore, food can be preserved without the loss of most of its fresh characteristics. Enzymes and micro-organisms are inactivated under high pressure. After an exposure time of 1 to 20 minutes, pressure is normalised. The pressure is applied isostatically, which implies that pressure distribution is uniform in the product. It is also important to note that

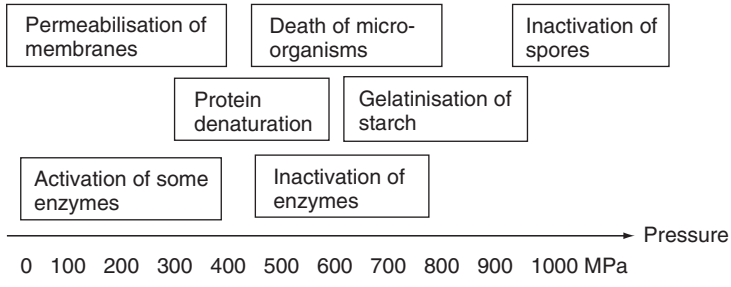


**Fig. 12.2** Effects of pressure on food properties.

HPP results only in minor temperature changes. Due to the adiabatic compression, a typical temperature change is in the range of 10–15°C.

At present, the technique is successfully used in Japan, United States and Europe for products such as citrus juices, guacamole, ham, oysters and rice.

### 12.5.2 *Pulsed electric field processing*

The preservation of bulk products by electrical impulses is an emerging technology that opens new perspectives for the food and pharmaceutical industries. This novel type of preservation method can be applied homogeneously through the product and is readily applicable to the pasteurisation of liquids at reduced temperatures. The application of PEF also allows foodstuffs to be treated at ambient temperatures. PEF treatment relies on the impact of intense electrical impulses on a microsecond time scale on microbial activity. When an electrical impulse is applied, the osmotic balance of micro-organisms present is disturbed and, under appropriate conditions, micro-organisms are inactivated. Reductions in plate counts exceeding five orders of magnitude can be established by a single impulse. In practice, the feasibility of PEF technology has already been demonstrated: preservation conditions were established at temperatures less than 30 °C resulting in inactivation of vegetative micro-organisms in combination with retention of the fresh characteristics.

### 12.5.3 *Novel processes and food allergenicity*

Novel processes may influence the allergenicity of food, mainly by altering the conformation and stability of proteins. In the process-parameter range used for food processing, high pressure alters proteins while pulsed electric fields have only a minor effect. High pressures can affect protein conformation and can lead to protein denaturation, aggregation or gelation, depending on the protein system, the applied pressure, the temperature and the duration of the pressure treatment. Conformational changes occur due to the effect of pressure on packing, hydration and non-covalent interactions in proteins. In general, reversible effects are observed below 100–200 MPa (e.g. dissociation of polymeric structures into subunits) while above 200 MPa, non-reversible effects may include complete inactivation of enzymes and denaturation of proteins [55, 56]. Figure 12.3 shows a general phase diagram of the denaturation of proteins during

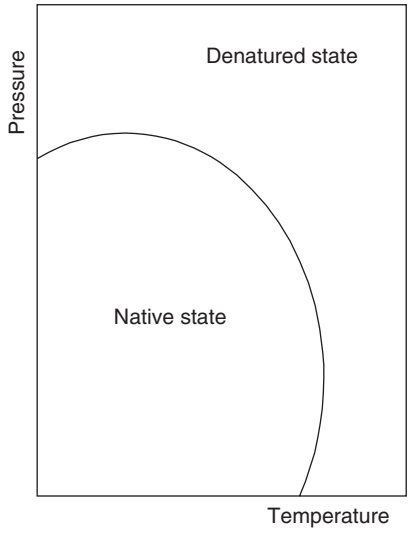


**Fig. 12.3** General scheme of the pressure–temperature phase diagram of proteins.

pressure/temperature treatment. The exact location of the native-denatured state transition depends on the protein and solvent used. High pressure can also affect Maillard reactions in food, depending on the pressure and temperature used [56].

A rice product with a lower allergenic potential is on the market in Japan, based on the preferential release of allergenic proteins by pressure treatment. When polished rice grains were immersed in water and pressurised at 100–400 MPa, a considerable amount of proteins was released. The major proteins released were identified as 16 kDa albumin, α-globulin and 33 kDa globulin, all of which are major rice allergens. The proposed mechanism is that the partial destruction of endosperm cells by pressurisation enhances the permeation of the surrounding solution into the rice grains. This results in the solubilisation of proteins and the subsequent diffusion from the endosperm cells to the surrounding solution. The removal of allergens by pressurisation only was insufficient. The allergenicity of the proteins was almost completely eliminated by pressurisation in the presence of proteolytic enzymes [57].

The recognition of ovalbumin by specific antibodies can also be decreased by high pressure treatment, due to modifications of the tertiary structure of the protein resulting in a significant decrease in the number of epitopes per unit mass of soluble protein. Ovalbumin represents >50% (w/w) of egg white protein and has a prominent role in determining the functional properties of egg albumin. The residual immunochemical reactivity of ovalbumin in albumin samples treated at 400 or 600 MPa for 5 minutes was 60% of that of the ovalbumin in untreated albumin [58]. It is interesting that these pressures can be used to stabilise egg albumin from a microbiological standpoint while retaining most of the technologically relevant properties of the material and decreasing the allergenicity.

Both thermal treatment and novel processes can result in increased accessibility of enzymes which can be used for reducing the allergenicity. An example of this is the

combination of high pressure with thermolysin on whey proteins for the specific elimination of beta-lactoglobulin which is a major allergen causing milk allergy. Beta-lactoglobulin is less resistant to pressure than alpha-lactalbumin. Pressures of 150 MPa caused partial denaturation of beta-lactoglobulin, making it accessible to thermolysin and resulting in degradation of beta-lactoglobulin without affecting alpha-lactalbumin [59].

However, high pressure treatment (600 MPa, 20°C) was not sufficient to destroy the IgE-binding capacity of the Api g 1 allergen of celery [8].

Reports on the impact of PEF on food allergenicity are scarce, but treatment of celery with 10 kV at 50 Hz, did not destroy the IgE-binding capacity of the Api g 1 allergen, nor did γ-irradiation (10 kGy) [8].

## 12.6 Future directions

Various processing techniques have been applied successfully to reduce the allergenicity of foods. Foods with decreased allergenicity may offer relief to considerable groups of sensitive consumers, as demonstrated by the soybean case [47] and possibly also with peanuts [45]. In this latter case, it should be realised that the differences in the prevalence of registered peanut allergy may also be caused by the incomparability in the health care systems in the United States and in China, and by differences in diagnostic and registration systems.

A wide variety of processing techniques, ranging from molecular biological via enzymatic to physical, have been applied in attempts to reduce allergenicity. The lack of generic approaches, and the highly empirical nature of the research, partially reflects the lack of structural data on allergens and allergenic epitopes. The development of more generic approaches can be accelerated by integrating biological and biochemical data on food allergens into food technology [60]. The latter discipline will generate essential data on the processing behaviour and stability of (allergenic) proteins and epitopes. The former disciplines are now generating vast data collections on protein structure and its genetic background, through the rapid developments in genomics, proteomics and bioinformatics. Proteins, and in particular their epitopes, can be modified without affecting their essential functions [5, 47], while minimal modifications may have great impacts on IgE recognition by epitopes [43, 44]. This opens the perspective for *in silico* screening of germplasm collections, to identify crop varieties with modified (i.e. lowered) allergenicity. As a direct spin-off of increased knowledge of the genomes of various agricultural crops, it can be anticipated that specific combinations of enzymes (proteases or oxidases) can be selected that may contribute to the modification of allergenic epitopes, and thus to reduction of their IgE binding. Additionally, the rapid developments in bioinformatics will result in more detailed knowledge of the factors that determine the stability of secondary, tertiary and quaternary structures of proteins, including those of allergens. This will allow the development of more specific (physical) processing methods, that more selectively process (i.e. destroy) allergenic structures.

However, as also stated in Section 12.3.4.1, it is doubtful whether processing will be sufficiently effective to completely remove allergenicity in all cases. In particular,

for allergies with severe symptoms, the introduction of rapid assay systems to monitor the allergenicity of foods will be an important aid for the food processing industry to safeguard integral production chains, from harvest, through storage to product development. A better correlation of the results of these *in vitro* systems and analytical data that are provided by, for example, skin prick tests or double-blind placebo-controlled food challenge tests will strongly improve their predictive value. This correlation can also be improved by application of the most suitable antibodies, e.g. pooled IgE-containing sera, in such assay systems.

Food markets are becoming increasingly consumer driven and the current desire to have food products that better resemble *fresh* products stimulates the development of a whole range of novel, minimal, processing techniques. Such minimal processing may have a reduced capacity to change the structure of proteins, and thus their potential allergenicity. Additionally, the current popularity of products that are obtained by organic farming, together with the biological function of many allergens as pathogenesis-related proteins, may also have impacts on the prevalence of allergies to plant-derived foods.

## References


1. Burks, A.W. & Stanley, J.S. (1998) Food allergy. *Curr Opin Pediat* **10**, 588–593.
2. Meredith, S. (ed.) (1999) European Allergy White Paper Update. Brussels: the UCB Institute of Allergy.
3. Jansen, M. & Brussaard, T. (2001) Voedselallergieën in Europa. *VMT* **34**(21), 11–13.
4. Taylor, S.L. & Hefle, S.L. (2001) Food allergies and other food sensitivities. *Food Technol* **55**, 68–83.
5. Soler-Rivas, C. & Wichers, H.J. (2001) Impact of (bio)chemical and physical procedures on food allergen stability. *Allergy* **56** (Suppl 67), 52–55.
6. Davis, P.J., Smales, C.M. & James, D.C. (2001) How can thermal processing modify the antigenicity of proteins? *Allergy* **56** (Suppl 67), 56–60.
7. Lehrer, S.B., Horner, W.E. & Rees, G. (1996) Why are some proteins allergenic? Implications for biotechnology. *Cr Rev Food Sci Nutr* **36**, 553–564.
8. Jankiewicz, A., Baltes, W., Bögl, K.W. *et al.* (1997) Influence of food processing on the immunochemical stability of celery allergens. *J Sci Food Agr* **75**, 359–370.
9. Taylor, S.L. & Lehrer, S.B. (1996) Principles and characteristics of food allergens. *Cr Rev Food Sci Nutr* **36**(S), S91–S118.
10. Hefle, S.L. (1996) The chemistry and biology of food allergens. *Food Technol* **50**, 88–92.
11. Matsuda, T. & Nakamura, R. (1993) Molecular structure and immunological properties of food allergens. *Trends Food Sci Tech* **4**, 289–293.
12. Taylor, S.L., Lemanski, R.F., Bush, R.K. & Busse, W.W. (1987) Food allergens: structure and immunologic properties. *Ann Allergy* **59**, 93–99.
13. Daul, C.B., Morgan, J.E., Waring, N.P., McCants, M.L., Hughes, J. & Lehrer, S.B. (1987) Immunological evaluation of shrimp-allergic individuals. *J Allergy Clin Immunol* **80**, 716–722.
14. Barnett, D., Bonhan, B. & Howden, W.E.H. (1987) Allergic cross-reactions among legume foods-*in vitro* study. *J Allergy Clin Immunol* **79**, 433–438.
15. Dreborg, S. & Foucard, T. (1983) Allergy to apple, carrot and potato in children with birch pollen allergy. *Allergy* **38**, 167–172.
16. Holm, J., Bærentzen, G., Gajhede, M. *et al.* (2001) Molecular basis of allergic cross-reactivity between group 1 major allergens from birch and apple. *J Chromatogr B* **756**, 307–313.
17. Fernandez-Rivas, M. & Cuevas, M. (1999) Peels of Rosaceae fruits have a higher allergenicity than pulps. *Clin Exp Allergy* **29**, 1239–1247.
18. Karamloo, F., Scheurer, S., Wangorsch, A., May, S., Haustein, D. & Vieths, S. (2001) Pyr c1, the major allergen from pear (*Pyrus communis*), is a new member of the Bet v 1 allergen familiy. *J Chromatogr B* **756**, 281–293.
19. Kinder, H. (1999) Die Typ 1 Allergie gegen Mangofrüchte unter Berücksichtigung von Kreuzreaktionen [thesis]. University of Hamburg, Germany.

20. Wigotzki, M. (2001) Einfluß der technologischen Verarbeitung auf das allergene Potential der birkenpollenassozierten Lebensmittel Haselnuß, Apfel und Karotte [thesis]. University of Hamburg, Germany.
21. Mills, E.N.C., Madsen, C., Shewry, P.R. & Wichers, H.J. (2001) Plant food protein allergens-the role of structure and function in allergenic potential. *Food Allergy Intol* **2**, 194–209.
22. Brenna, O., Pompei, C., Ortolani, C., Pravettoni, V., Farioli, L. & Pastorello, E.A. (2000) Technological processes to decrease the allergenicity of peach juice and nectar. *J Agric Food Chem* **48**, 493–497.
23. Hage, S.H. (1993) Immunoassays. *Anal Chem* **65**, 420–424.
24. Allen, J.C. (1989) Immunoassay kits for food analysis. In: *Resources and Applications of Biotechnology: the New Wave*, Greenshields, R. (ed.). The MacMillan Press Ltd, Hampshire and London, pp. 383–388.
25. Gazzaz, S.S., Rasco, B.A. & Dong, F.M. (1992) Application of Immunochemical assays to food analysis. *Cr Rev Food Sci Nutr* **32**, 197–229.
26. Rittenburg, J.H. & Grothaus, G.D. (1992) ImmunoAssays: formats and applications. In: *Food Safety and Quality Assurance: Applications of Immunoassay Systems*. Elsevier Applied Science, London and New York.
27. Pohland, A.E., Trucksess, M.W. & Page, S.W. (1990) Immunoassays in food safety applications – developments and perspectives. In: *Immunochemical Methods for Environmental Analysis*, Van Emom, J.M. & Mumma, R.O. (eds). ACS Symposium series, American Chemical Society, Washington, USA, Vol. 442, pp. 38–50.
28. Samarajeewa, U., Wei, C.I., Huang, T.S. & Marshall, M.R. (1991) Application of immunoassays in the food industry. *Food Sci Nutr* **29**, 403–434.
29. Morgan, M.R.A., Smith, C.J. & Williams, P.A. (eds) (1992) In: *Food Safety and Quality Assurance: Applications of Immunoassay Systems*. Elsevier Applied Science, London and New York.
30. Oh, S. (1994) Immunosensors for food safety. *Trends Food Sci Tech* **4**, 98–103.
31. Van Amerongen, A., Van Loon, D., Berendsen, L.B.J.M. & Wichers, J.H. (1994) Quantitative computer image analysis of a human Chorionic Gonadotropin colloidal carbon dipstick assay. *Clin Chim Acta* **229**, 67–75.
32. Aldus, C.F., Ariëns, R.M.C., Wichers, J.H., Peck, M.W., Van Amerongen, A. & Wyatt, G.M. (2003) Principles of some novel rapid dipstick methods for detection and characterisation of verotoxigenic *E. coli* (VTEC). *J Appl Microbiol* **94**, 1–10.
33. Peters, A.M., Haagsma, N., Gensch, K.-H. & Van Amerongen, A. (1996) Production and characterization of polyclonal antibodies against bitter sesquiterpene lactones of chicory (*Cichorium intybus* L.). *J Agric Food Chem* **44**, 3611–3615.
34. Van Amerongen, A., Plasman, H.H., Kuperus, D. *et al.* (1994) Design of peptides with improved affinities for anti-human Chorionic Gonadotropin monoclonal antibodies. *Peptide Res* **7**, 83–90.
35. Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L. & Fuchs, R.L. (1996) Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Cr Rev Food Sci Nutr* **36**(S), S165–S186.
36. Wichers, H.J., Peres, S. & Van Amerongen, A. Role of oxidizing enzymes in the allergenicity of apple. Submitted.
37. Dutau, G. & Rancé, F. (2001) Peanut allergy. *Rev Fr Allerg Immun Clin* **41**, 187–198.
38. Burks, A.W., Shin, D., Cockrell, G., Stanley, J.S., Helm, R.M. & Bannon, G.A. (1997) Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* **245**, 334–339.
39. Koppelman, S.J., Bruijnzeel-Koomen, C.A.F.M., Hessing, M. & De Jongh, H.H.J. (1999) Heat-induced conformational changes of Ara h 1, a major peanut allergen, do not affect its allergenic properties. *J Biol Chem* **274**, 4770–4777.
40. Maleki, S.J., Kopper, R.A., Shin, D.S. *et al.* (2000) Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J Immunol* **164**, 5844–5849.
41. Taylor, S.L., Busse, W.W., Sachs, M.I., Parker, J.L. & Yunginger, J.W. (1981) Peanut oil is not allergenic to peanut-sensitive individuals. *J Allergy Clin Immunol* **68**, 372–375.
42. Hefle, S.L. & Taylor, S.L. (1999) Allergenicity of edible oils. *Food Technol* **53**, 62–70.
43. Van Amerongen, A., Beckers, P.J.A., Plasman, H.H. *et al.* (1992) Peptides reactive with a transmission blocking monoclonal antibody against *P. falciparum* pfs25: 2000-fold affinity increase by PEPSCAN-based amino acid substitutions. *Peptide Res* **5**, 269–274.
44. Burks, A.W., Helm, R.M., Cockrell, G. *et al.* (1999) Tertiary structure of peanut allergen Ara h 1. PTC Patent Application, Board of Trustees of the University of Arkansas.
45. Beyer, K., Morrow, E., Li, X.-M. *et al.* (2001) Effects of cooking methods on peanut allergenicity. *J Allergy Clin Immunol* **107**, 1077–1081.
46. Burks, A.W., Williams, L.W., Helm, R.M., Thresher, W., Brooks, J.R. & Sampson, H.A. (1991) Identification of soy protein allergens in patients with atopic dermatitis and positive soy challenges:

determination of change in allergenicity after heating or enzyme digestion. In: *Nutritional and Toxicological Consequences of Food Processing*, Friedman, M. (ed.). Plenum Press, New York, pp. 295–307.
47. Ogawa, T., Samoto, M. & Takahashi, K. (2000) Soybean allergens and hypoallergenic soybean products. *J Nutr Sci Vitaminol* **46**, 271–279.
48. Yamanishi, R., Tsuji, H., Bando, N. *et al*. (1996) Reduction of allergenicity of soybean by treatment with proteases. *J Nutr Sci Vitaminol* **42**, 581–587.
49. Tanabe, S., Arai, S. & Watanabe, M. (1996) Modification of wheat flour with bromelain and baking hypoallergenic bread with added ingredients. *Biosci Biotech Biochem* **60**, 1269–1272.
50. Watanabe, M., Tanabe, S., Suzuki, T., Ikezawa, Z. & Arai, S. (1995) Primary structure of an allergenic peptide occurring in the chymotryptic hydrolysate of gluten. *Biosci Biotech Biochem* **59**, 1596–1597.
51. Watanabe, M., Yoshizawa, T., Miyakawa, J. *et al*. (1990) Quality improvement and evaluation of hypoallergenic rice grains. *J Food Sci* **55**, 1105–1107.
52. Watanabe, M. (1993) Hypoallergenic rice as a physiological functional food. *Trends Food Sci Tech* **4**, 125–128.
53. Tada, Y., Nakase, M., Adachi, T. *et al*. (1996) Reduction of 14–16 kDa allergenic proteins in transgenic rice plants by antisense gene. *FEBS Lett* **91**, 341–345.
54. Burks, A.W. & Fuchs, R.L. (1995) Assessment of the endogenous allergens in glyphosate-tolerant and commercial soybean varieties. *J Allergy Clin Immunol* **96**, 1008–1010.
55. Messens, W., Van Camp, J. & Huyghebaert, A. (1997) The use of high pressure to modify the functionality of food proteins. *Trends Food Sci Tech* **8**, 107–111.
56. Balny, C. & Masson, P. (1993) Effects of high pressure on proteins. *Food Rev Int* **9**, 611–628.
57. Kato, T., Katayama, E., Matsubara, S., Omi, Y. & Matsuda, T. (2000) Release of allergenic proteins from rice grains induced by high hydrostatic pressure. *J Agric Food Chem* **48**, 3124–3129.
58. Iametti, S., Donnizzelli, E., Pittia, P., Robere, P.P., Squarcina, N. & Bonomi, F. (1999) Characterization of high-pressure-treated egg albumin. *J Agric Food Chem* **47**, 3611–3616.
59. Okamoto, M., Hayashi, R., Enomoto, A., Kaminogawa, S. & Yamauchi, K. (1991) High-pressure proteolytic digestion of food proteins: selective elimination of ß-lactoglobulin in bovine milk whey concentrate. *Agric Biol Chem* **55**, 1253–1257.
60. Moneret-Vautrin, D.A. (1998) Modifications of food allergenicity linked to food technologies. *Allergie et Immunologie* **30**, 9–13.

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